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TUMOUR MARKERS AS PROGNOSTIC FACTORS OF SURVIVAL OF GASTRIC CANCER PATIENTS

by

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Academic Dissertation

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II Wiksten J-P, Lundin J, Nordling S, Haglund C. The prognostic value of p27 in gastric cancer. *Oncology.* 2002;**63(2):**180-184.

III Wiksten J-P, Lundin J, Nordling S, Lundin M, Kokkola A, von Boguslawski K, Haglund C. Tenascin-C expression correlates with prognosis in gastric cancer. *Oncology*. 2003;**64(3):**245-250.

IV Wiksten J-P, Lundin J, Nordling S, Lundin M, Kokkola A, Stenman U-H, Haglund C. High tissue expression of tumour-associated trypsin inhibitor (TATI) associates with a more favourable prognosis in gastric cancer. *Histopathology.* 2005;**46:**380-388.

V Wiksten J-P, Lundin J, Nordling S, Kokkola A, Haglund C. Comparative prognostic value of a panel of tissue tumor markers in gastric cancer. Submitted.

2. ABBREVIATIONS

ABC	avidin-biotin complex
AB-PAS	alcian blue periodic acid-Schiff
bcl-2	proto-oncogene bcl-2
CagA	cytotoxin-associated gene A
CI 95%	confidence interval at 95% level
Cdk	cyclin-dependent kinase
CDKN	cyclin-dependent kinase inhibitor
Cip1	cyclin-dependent kinase-interacting protein
CV	coefficient of variation
D	dissection of lymph nodes
DI	DNA index
DNA pl	DNA ploidy
ECM	extracellular matrix
EGF	epidermal growth factor
GISTs	gastrointestinal stromal tumours
GAGs	glycosaminoglycans
HE	haematoxylin and eosin
HLA	human leukocyte antigen
H. pylori	*Helicobacter pylori*
IL	interleukin
KIP1	cyclin-dependent kinase inhibitor protein-1
MAb	monoclonal antibody
MALT	mucosa-associated lymphoid tissue
Mdm2	mouse double minute 2
NS	non-significant
O/N	overnight
p21	nuclear phosphoprotein p21
p27	nuclear phosphoprotein p27
p53	nuclear phosphoprotein p53
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pM	pathological metastasis status
pN	pathological nodal status
PSTI	pancreatic secretory trypsin inhibitor
pT	pathologic tumour penetration depth
RH	relative hazard
r_s	Spearman's rank correlation coefficient
R/T	room temperature
SDC1	syndecan-1
SPF	S-phase fraction
SPINK1	serine peptidase inhibitor, Kazal type 1
TATI	tumour-associated trypsin inhibitor
TNC	tenascin-C
TNM	tumour, node, metastasis
TGFβ	transforming growth factor-beta
TP53	tumour protein p53
UICC	Union Internationale Contre le Cancer
Waf1	wild-type p53-activated fragment 1

3. ABSTRACT

The incidence of gastric cancer in the last decades has declined rapidly in the industrialised countries. Worldwide, however, gastric cancer is still the second most common cause of cancer death. Although surgery is currently the most effective treatment, the rapid progress in adjuvant chemotherapy and radiation therapy requires a re-evaluation of prognosis assessment. The TNM staging system of the UICC is ubiquitously used; it groups patients by decreasing survival times from stage I to stage IV based on the spread of disease, i.e., depth of tumour penetration (T), extent of spread to regional lymph nodes (N), and the presence or absence of distant (M) metastases. This is by far the most consistent prognostic classification system today. However, even within the stage groups there are patients that follow a varying course of disease. Our knowledge of the molecular differences between tumours of the same stage and morphology has been accumulating over the years and methods for a more accurate assessment of the phenotype of neoplasias are of value when evaluating the prognosis of individual patients with gastric cancer.

In this study, the immunohistochemical expression of tumour markers involved in different phases in tumourigenesis was examined. The aim was to find new markers which could provide prognostic information in addition to what is provided by the TNM variables. A total of 337 specimens from the primary tumour of patients who underwent surgery for gastric cancer were collected and the immunohistochemical expression of seven different biomarkers was analysed. DNA ploidy and S-phase fraction (SPF) was assessed by flow cytometry. Finally, all biomarkers and clinicopathological prognostic factors were combined and evaluated by a multivariate Cox regression model to elucidate which specific factors provide independent prognostic information.

By univariate survival analysis the following variables were significant prognostic factors: epithelial and stromal syndecan-1 expression, stromal tenascin-C expression, expression of tumour-associated trypsin inhibitor (TATI) in cancer cells, nuclear p53 expression, nuclear p21 expression, DNA ploidy, and SPF. By multivariate survival analysis adjusted for all available clinicopathological and biomolecular variables, p53 expression, p21 expression, and DNA ploidy emerged as independent prognostic

biomarkers, together with penetration depth of the tumour, presence of regional nodal metastases, surgical cure of the cancer, and age of the patient at the time of diagnosis.

4. INTRODUCTION

Knowledge of the outcome after surgery of patients with gastric cancer is crucial for subsequent planning of treatment and follow-up. Although the incidence of gastric cancer has rapidly declined in the industrialised countries after the Second World War, gastric cancer is still the fourth most common cause of cancer death in Finland and the second most common worldwide, preceded only by lung cancer. The incidence of gastric cancer in Finland in 2003 was 9.6/100,000 for men (424 new cases) and 5.2/100,000 for women (347 new cases), which accounts for 3.4 % of all cancers in men and for 2.9 % in women (Finnish Cancer Registry, 2005). Mortality due to gastric cancer among men was 7.1/100,000 (314 deaths) and among women 3.3/100,000 (248 deaths), which makes gastric cancer the 4th and 6th most common killer cancer, accounting for 5.8 % and 4.9 % of all cancer deaths, respectively. The predicted 5-year relative survival rates of patients diagnosed with gastric cancer in 1999 – 2001 are 27 % for men and 31 % for women (Finnish Cancer Registry, 2005). Prognosis is worse only for cancer of the pancreas, liver, gall bladder, oesophagus, and lung and trachea. A difference in the incidence of gastric cancer by socioeconomic status is evident for both men and women: the risk is higher in the lowest socioeconomic group than in the highest. This may partly be explained by dietary factors and the prevalence of *Helicobacter pylori* infection. The incidence of gastric cancer rises with increasing age: in the age group 25 – 29 years it is 0.2/100,000 standardised person years and in the age group of 85 and older it is 177.8.

The only potentially curative treatment of gastric cancer today is total surgical removal of the primary tumour and the affected lymph nodes. However, a large number of clinical trials are currently ongoing in an effort to develop more effective adjuvant therapies. Before a patient is decided to be put on adjuvant therapy, it would naturally be most important to know the risk of disease recurrence and prognosis. This would make it possible to target treatments accurately to patients that would benefit and to avoid potentially harmful treatments of patients who would not benefit. In addition to improved patient care also considerable economical advantages are involved.

The most commonly used and validated prognostic tool today is the TNM-stage classification system, which focuses on the anatomic extent of the disease at the time of detection. It is based on a collective evaluation of the depth of tumour penetration

(pT), regional lymph node involvement (pN) and presence or absence of distant metastases (pM). More accurate prognostic tools are, however, needed. Patients with stage I cancer have a favourable prognosis with a 5-year survival of 90 – 95 %. Some of the patients will, however, ultimately die of the cancer and they would benefit from further treatments. Stage IV patients have an unfavourable prognosis with 5-year survival of 0 – 30 %, but, again, a considerable number of patients survive also in this subgroup. The survival for stage II-III patients is much more variable and more accurate prognostic tools are urgently needed especially for these patients.

Development of malignant neoplasia is a multistep process, which involves several disturbances in the control mechanisms of cellular growth. It is possible to detect molecular changes which differ between malignant and healthy cells. Immunohistochemistry is a well established method to study the expression of specific antigens in tissues and can be used to examine cancer-specific expression patterns. Flow cytometric analysis of the DNA ploidy and S-phase fraction is another well established method to assess the amount of DNA in a cell suspension and the level of chromosomal derangements in a specimen.

The prognostic value of the different biomarkers has generally been studied one at a time. Prognosis-setting could be improved by a model that would use information from several independent prognostic factors. In this study exploratory analyses of single new biomarkers were performed, and attempts were made to find the most accurate combination of prognostic variable markers with regard to patient outcome. The study was conducted on patients with gastric adenocarcinoma only, to be distinguished from gastric lymphoma and leiomyosarcoma, which have different tumour biology. Of all gastric tumours about 90 to 95 % are adenocarcinomas. This study was performed to identify new prognostic tumour markers and to combine them into a prognostic model in order to improve the prediction of the outcome of patients with gastric cancer.

5. REVIEW OF THE LITERATURE

5.1 Types of gastric cancer

The stomach consists of five different layers: mucosa, submucosa, muscularis, subserosa, and serosa. Malignancies may develop in any of these layers. The most common of the gastric cancers is the adenocarcinoma, which makes up 90 – 95 % of all gastric malignancies. Adenocarcinoma arises from the glandular epithelial cells of the inner mucosal layer of the stomach. Other types of gastric malignancies are mainly lymphomas and gastrointestinal stromal tumours (GISTs). Lymphomas are rare tumours of the lymphatic system, of which the mucosa-associated lymphoid tissue (MALT) lymphomas are the most common in the stomach. Rarely, neuroendocrine tumours arise in the stomach. Occasionally the stomach is the site for metastasis, usually from an adenocarcinoma originated from the pancreas, breast, or ovary.

The stomach is composed of the cardia, which is the mouth of the stomach from oesophagus, and caudally leftwards of the fundus. The major part of the stomach consists of the body or corpus, with the lesser curvature to the upper right and the greater curvature to the lower left. The mucosa of the fundus and corpus is folded (rugae); the distal third of the stomach is devoid of such folds. The antrum is marked by the incisura angularis proximally and by the pyloric region distally. Roughly 15 % of the gastric cancers develop in the upper, 40 % in the middle, and 40 % in the distal part of the stomach. About 10 % of the cancers engage more than one of the parts at the time of diagnosis. The incidence of gastric cardia cancer has increased in the industrialised countries (Botterweck et al., 2000; Kocher et al., 2001; Blaser et al., 2002).

5.2 Epidemiology

Gastric adenocarcinoma is an important cause of mortality and morbidity worldwide. It is the second most common cause of cancer-related deaths with approximately 700,000 deaths in 2002 (Boyle, 1997; Torrado et al., 2001; Roder, 2002). The incidence has rapidly declined in the industrialised countries over the last 5 decades but it is still high in countries like Japan (59.9 men and 23.8 women per 100,000 persons in 1993-97), Korea, China, Central and South American, and the East European countries. In 1957 the number of new gastric cancer cases in Finland was 1,876 but in 2003 only 783 (Finnish Cancer Registry, 2005) (Figure 1). The incidence of gastric cancer increases

with age and is highest in the age group above 50. The median age at diagnosis is about 65 years. There were on average 764 new cases of gastric cancer diagnosed annually in 1999 – 2004 per year, with 603 (79 %) of the patients being 60 years or older (Finnish Cancer Registry, 2005). Gastric cancer is 1.5 – 2.0 times more prevalent among men than women (Finnish Cancer Registry, 2005).

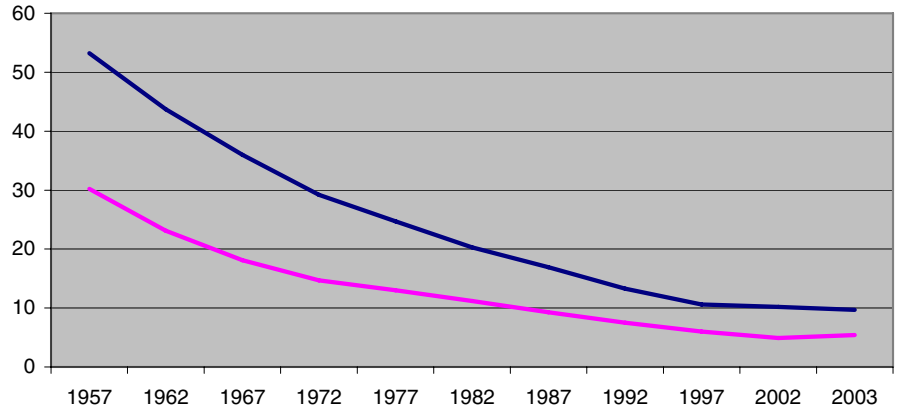

Figure 1. Incidence per 100,000 inhabitants of gastric cancer in Finland from 1957 to 2003. Men (blue line) and women (red line) (Finnish Cancer Registry, 2005).

Mortality related to gastric cancer is high. In 2003, gastric cancer was the fourth most common cause of cancer-related deaths among Finnish men, preceded only by lung, prostate and pancreas cancer. There were 314 deaths (5.8 % of all cancer deaths for men, 7.1/100,000 persons) in 2003. In women gastric cancer was the sixth most common cause of cancer related deaths, preceded by breast, lung, pancreas, colon, and ovarian cancer (4.9 % of all, 3.3/100,000 persons) (Finnish Cancer Registry, 2005). The predicted 5-year survival of men diagnosed with gastric cancer from 1999 to 2001 in Finland is 27 % and of women 31 % (Finnish Cancer Registry, 2005).

There is some evidence that patients with gastric cancer of Asian descent have a better prognosis. This may be due to a greater frequency of early stage disease, different staging classification, earlier detection of disease due to screening, more distant tumour location, and perhaps more extensive surgery as is customary in Asian

hospitals (Hundahl et al., 1996; Noguchi et al., 2000). There seem also to be differences in disease patterns between patients with different ethnicity within Western treatment centres. Japanese Americans have an advantage in stage-stratified gastric cancer survival mainly because they have fewer proximal tumours, a lower male-to-female gastric cancer ratio, and less often undergo adjacent organ resection (Hundahl et al., 2000). Patients of Asian descent tend also to be younger, precent with less distant metastases, and more likely undergo curative resection (Theuer et al., 2000; Schwarz et al., 2002; Gill et al., 2003).

5.3 Aetiology and risk factors

The mechanism of how gastric cancer develops is unknown. Of all gastric cancers, only 1 % is hereditary (Caldas et al., 1999), and when this is the case, the patient is usually younger than 40 years (Caldas et al., 1999). Familial hereditary gastric polyposis with a high incidence of gastric cancer has been described (Seruca et al., 1991). Chronic atrophic gastritis followed by intestinal metaplasia is a precancerous lesion linked to development of malignant gastric neoplasia. Pernicious anaemia is, in turn, linked to chronic atrophic gastritis.

An important aspect of gastric carcinogenesis is the infection of *Helicobacter pylori*, which gives rise to a variety of inflammatory responses. This variation is partly due to the genetic variation of *H. pylori* and partly to different immunological responses of the patient (Kaminishi, 2005). Several factors as salty food, nitrite and N-nitroso compounds, lack of vitamin C, individual immunogenic response, interleukin-1 polymorphism, and *H. pylori* infection all interact over a long time span and may finally induce gastric cancer.

5.3.1 *Helicobacter pylori* infection

The *H. pylori* organism was found by Marshall and Warren in 1982 (Marshall & Warren, 1984). This bacterium has been closely linked to gastritis and the risk of gastric cancer among infected individuals is increased 2- to 9-fold (Eslick et al., 1999; Huang et al., 2003). Although all patients with *H. pylori* infection show signs of gastritis, only a small percent of them ultimately develop gastric ulcer and much less cancer. Based on epidemiologic findings WHO declared in 1994 that *H. pylori* is a carcinogen and constitutes a risk factor for gastric cancer. *H. pylori* infection,

especially the CagA-positive strain, is associated particularly with noncardia gastric adenocarcinoma, but the risk of gastric cancer among patients with CagA-negative *H. pylori* is also significantly increased when compared to uninfected persons (Parsonnet et al., 1997; Enroth et al., 2000; Nomura et al., 2002; Huang et al., 2003; Wu et al., 2003; Held et al., 2004; Machida-Montani et al., 2004; Lopez-Carrillo et al., 2004).

Smoking adds significantly to the raised risk of gastric cancer related to CagA positive *H. pylori* infection (Brenner et al., 2002). *H. pylori* infection does not seem to associate with adenocarcinoma of the gastric cardia (Wu et al., 2003; Huang et al., 2003; Ye et al., 2004). A great deal of the decrease in the incidence of gastric cancer can be contributed to better hygienic conditions, improved cold storage of food and antibiotics with activity against *H. pylori*. Eradication of *H. pylori* seems to reduce the incidence of gastric cancer, at least among people without precancerous lesions during the eradication (Hamajima et al., 2004; Wong et al., 2004; Kaminishi, 2005). Duodenal ulcer is strongly associated with *H. pylori* infection and associates with a reduced risk of intestinal metaplasia, which is considered to be a premalignant lesion in the stomach (Leung et al., 2004).

5.3.2 Hereditary and genetic factors

Families with several first-degree relatives with gastric cancer have a higher risk of gastric cancer. There may be a familiar predisposition if two first-degree relatives, one younger than 50 years, or at least 3 first-degree relatives irrespective of age have had gastric cancer. A relative risk of 2.6 has been reported between the risk of gastric cancer and a family history of gastric cancer (La Vecchia et al., 1992). Families with frequent gastric cancer at a young age usually develop the diffuse type of gastric cancer (Guilford et al., 1998; Gayther et al., 1998; Berx et al., 1998).

Individual immunological, genetic, and immunogenetic factors are important in the pathogenesis of gastric cancer. A genetic profile favouring a proinflammatory response increases the risk of gastric carcinoma (Shang & Pena, 2005; Zagari et al., 2004). For unknown reasons, blood group A may also be associated with an increased risk of gastric cancer (Hallstone & Perez, 1994). The risk of gastric cancer among *H. pylori* infected persons may also be associated with specific HLA genotypes and proinflammatory IL-1 polymorphisms (Magnusson et al., 2001; Rad et al., 2004; Li et

al., 2005). A family history of gastric cancer and CagA positive *H. pylori* infection has been related to a more than 8-fold risk of gastric carcinoma and a 16-fold risk of noncardia gastric carcinoma, when compared with uninfected subjects with no family history (Brenner et al., 2000). However, genetic mechanisms do not seem to play as important a role in gastric cancer as for colorectal cancer, and familial clustering of the disease might reflect rather environmental factors shared by family members than true genetic differences (Goldgar et al., 1994). Interestingly, persons with inherited genetic disorders, such as hereditary nonpolyposis colorectal cancer and the inherited breast cancer genes BRCA1 and BRCA2, may also be at a higher risk of developing gastric cancer (Ericson et al., 2004; Lorenzo Bermejo et al., 2004).

5.3.3 Dietary and lifestyle factors

In a study on 38,576 atomic-bomb survivors in Hiroshima and Nagasaki there were 1,270 gastric carcinomas during the follow-up between 1980 and 1999 (Sauvaget et al., 2005). Exposure to ionizing radiation, male gender, age, and smoking were significantly associated with an increased risk of gastric cancer but no association with the consumption of fruit, vegetables, green tea, and soy products was observed (Sauvaget et al., 2005). Food rich in salt, salted meat, starch, and pickled food seem to be risk factors for gastric cancer, while diets rich in fruits and vegetables and low in salt and starch tend to have a protective effect (Sauvaget et al., 2003; De Stefani et al., 2004; Tsugane, 2005; Turkdogan et al., 2005). Smoking and overweight have been associated with an increased risk of gastric cardia cancer (Chow et al., 1998; You et al., 2000; Wu et al., 2001; Engel et al., 2003; Lindblad et al., 2005), and alcohol consumption with an overall risk of gastric cancer (Ji et al., 1996), but this has not been the case in all studies (Gammon et al., 1997; Wu et al., 2001; Engel et al., 2003; Barstad et al., 2005; Lindblad et al., 2005).

Hyperglycaemia is a risk factor for gastric cancer, especially among *H. pylori* positive subjects (Yamagata et al., 2005). Chilli peppers contain capsaicin and a high intake may also associate with an increased risk of gastric cancer (Lopez-Carrillo et al., 2003). On the other hand, protective effects have been reported from the intake of carotenes, lycopene, and vitamin C (Yuan et al., 2004), although controversial results are also reported (Lopez-Carrillo et al., 2004). Low levels of dietary vitamin C and *H. pylori* infection are associated with an increased risk of gastric cancer (You et al.,

2000). High levels of vitamin C inhibit the growth of *H. pylori* in vitro and in vivo (Zhang et al., 1997). Nitrite consumption may not (Lopez-Carrillo et al., 2004) or may (Engel et al., 2003; Bonney et al., 1987) be associated with the development of gastric cancer.

A low socioeconomic status has been linked to an increased risk of gastric cancer (Gammon et al., 1997; Tran et al., 2005), especially if associated with a low educational status, consumption of smoked, salted, fatty foods, and well water, cigarette smoking, poor intake of fresh fruits and vegetables, and poor hygiene (Fujino et al., 2002; Turkdogan et al., 2005).

5.3.4 Gastric stump after previous surgery for benign disease

Gastric stump cancer is a carcinoma that has developed in the gastric stump after partial gastrectomy for some benign gastric disease (Matsui et al., 2001). This condition may increase the risk of subsequent gastric cancer (Hansson et al., 2000), but this report has been challenged (Luukkonen et al., 1990). However, enteric bile reflux and *H. pylori* may act in synergy on cell proliferation in the stump (Leivonen et al., 1997). There is evidence that *H. pylori* is less strongly associated with cancer in the stump than otherwise and that intestinal reflux and subsequent reactive gastropathy are more important reasons for carcinogenesis in the gastric stump (MacDonald et al., 2001; Johannesson et al., 2003).

5.3.5 Pernicious anaemia

Pernicious anaemia and achlorhydria follow type A gastritis affecting the body and fundus of the stomach (Toh et al., 1997). Pernicious anaemia is considered to be a risk factor for non-cardia gastric cancer (Ye et al., 2003; Brinton et al., 1989; Hsing et al., 1993; Karlson et al., 2000; Mellemkjaer et al., 1996).

5.3.6 Polyps

Gastric polyps are proliferative or neoplastic lesions of the gastric mucosal layer, protruding into the lumen, and they occur in 2 – 3 % of all gastroscopies (Lau et al., 1998; Oberhuber & Stolte, 2000). Gastric polyps may be sporadic (Daibo et al., 1987; Oberhuber & Stolte, 2000; Park et al., 2001) or associated with polyposis syndromes, e.g., familial adenomatous polyposis coli, the Peutz-Jeghers syndrome, juvenile

polyposis, and Cowden's disease (Spigelman et al., 1989; Hofgartner et al., 1999; Oberhuber & Stolte, 2000). Depending on the type of polyps there is an increased risk (2 – 15 %) of gastric cancer (Daibo et al., 1987; Abraham et al., 2002).

5.3.7 Epstein-Barr virus

The Epstein-Barr virus cause mononucleosis and has been associated with some malignancies as Burkitt's lymphoma (zur Hausen et al., 1970), nasopharyngeal carcinoma (zur Hausen et al., 1970), and gastric cancer (Shibata et al., 1992; Neugut et al., 1996). Gastric cancers caused by the Epstein-Barr virus tend to be slowly growing and not very invasive.

5.3.8 Ménétrier's disease

Ménétrier's disease is a form of gastritis called giant hypertrophic gastropathy. It is characterised of large folding of the stomach, low acid production and hypoproteinaemia. The increased risk of gastric cancer in Ménétrier's disease is mainly anecdotal, since the disease is a rarity (Fieber & Rickert, 1981; Wood et al., 1983; Simson et al., 1987; Johnson et al., 1995).

5.4 Treatment

5.4.1 Curative resection

Curative surgery (R0 resection according to UICC) is defined as complete removal of all gastric cancer with a resection margin devoid of malignancy, both macroscopically and microscopically (Hermanek & Sobin, 1987). A resection with residual microscopic disease is called R1 and a resection with gross residual disease R2. Patients with local disease who undergo R0 resection will most likely survive their disease. Several large studies on the prognosis of gastric cancer have shown that curative surgical treatment as assessed by the surgeon is an important, independent prognostic factor (Kim et al., 1998; Kim et al., 2001).

Gastrectomy procedures include 1) total gastrectomy, 2) subtotal gastrectomy for tumours of the antrum or distal body, and 3) proximal subtotal gastrectomy or oesophago-gastrectomy for cancer of the cardia. Total gastrectomy is not considered

necessary if UICC R0 resection can be performed and there is no evidence of microscopic or macroscopic residual cancer (Brennan et al., 2005).

5.4.2 Lymph node dissection

Radical and curative resection requires also total resection of all involved lymph nodes. A D0 dissection is defined as gastric resection with incomplete removal even of the perigastric (N1) lymph nodes; D1 dissection is lymphadenectomy of the perigastric lymph nodes adjacent to the tumour (N1); D2 dissection includes the nodes along the stomach and the nodes along the common hepatic artery, left gastric artery, lienal artery, and around the celiac axis (N1 and N2); D3 dissection is lymphadenectomy of the N1, N2, and N3 lymph nodes along the portal vein, superior mesenteric vein and artery, and the retropancreatic nodes; and D4 dissection is a superextended lymphadenectomy that includes the nodes mentioned above together with the N4 lymph nodes, including the para-aortic nodes as far caudally as the inferior mesenteric artery, the nodes along the porta hepatis and along the vena cava. Extended (D2) lymph node dissection is the current standard of care in Japan and it is considered safe when performed by experienced surgeons (Kodama et al., 1981; Sano et al., 2004).

Even for patients with early gastric cancer extended D2 lymphadenectomy should be performed if there is a high risk of lymph node metastases with larger tumours or lymphatic or blood vessel invasion (Kim et al., 2004; Hyung et al., 2004). Extended lymph node (D2) dissection for patients with histologically node-negative gastric cancer with advanced T stage is associated with improved survival (Harrison et al., 1998; Siewert et al., 1998). Extended D2 lymphadenectomy is reported to be beneficial when compared to D1 dissection while morbidity and mortality are unaffected (Roukos et al., 1998; Lewis et al., 2002; Degiuli et al., 2004), whereas splenectomy and pancreatectomy are linked with increased morbidity but no survival benefit (Schmid et al., 2000; McCulloch et al., 2004). Previous studies in European centres reported, however, that D2 resection was associated with increased operative mortality and postoperative complications (Bonenkamp et al., 1995; Cuschieri et al., 1996).

5.4.3 Adjuvant therapy

A large number of adjuvant chemotherapy trials over the past decades have been conducted, but the results have not been encouraging. A study involving 556 patients

with resected adenocarcinoma of the stomach or the gastroesophageal junction randomly assigned patients to surgery with postoperative chemoradiotherapy or surgery alone. The adjuvant treatment consisted of fluorouracil and leucovorin, followed by radiotherapy and one month later of two additional cycles of fluorouracil and leucovorin. The median overall survival was 27 months in the surgery-only group and 36 months in the chemoradiotherapy group (RH = 1.35, 95 % CI = 1.09 – 1.66; p = 0.005). There was a significant reduction in the frequency of relapses in the chemoradiotherapy group ($p < 0.001$). Toxic, therapy-associated effects occurred in 41 % and 3 patients (1 %) died from toxic effects in the chemoradiotherapy group (Macdonald et al., 2001).

In another study involving 260 patients with curative resection for stage II to stage IVM0 gastric cancer, the patients were randomised to postoperative adjuvant chemotherapy with 5-fluorouracil and cisplatin or to surgery alone. The 5- and 7-year overall survival rates were 41.9 % and 34.9 % in the control group compared to 46.6 % and 44.6 % in the chemotherapy group ($p = 0.22$). Only 48.8 % of the patients received more than 80 % of the planned dose as a result of toxicity. The rate of recurrence was reduced by 30 % in the chemotherapy group ($p = 0.03$) (Bouche et al., 2005).

According to a recent Cochrane review, chemotherapy significantly improves the survival of patients with advanced gastric cancer in comparison to best supportive care (Wagner et al., 2005). Combination chemotherapy seems to improve survival further compared to single-agent 5-fluorouracil, but the effect is modest. The results on survival have been best on combination chemotherapy regimens containing 5-fluorouracil, an anthracycline and cisplatin: half of the patients are alive at 7 months of diagnosis compared to 5.9 months for those on monotherapy. However, combination therapies are more toxic than monotherapies. Combination chemotherapy with epirubicin, cisplatin and continuous infusion of 5-fluorouracil is tolerated best.

5.5 Prognostic factors

5.5.1 TNM classification

The tumour-node-metastasis (TNM) stage classification of Union Internationale Contre le Cancer (UICC) is the most widely recognised system to classify the spread of cancers – also gastric cancer – and it predicts patient outcome (Kirkwood et al.,

1997; Buonadonna et al., 2003). The TNM classification classifies patients into six different groups by depth of penetration into the gastric wall of the primary tumour (pT), spread of malignant cells to local lymph nodes (pN), and the presence or absence of distant metastases (pM) (Hermanek & Sobin, 1987; Sobin & Wittekind, 2002). A clinical classification (cTNM or TNM) is used to describe the pretreatment TNM status of the patient and a pathological classification (pTNM) to describe the postsurgical histopathological status; both are used clinically. The former is used for selecting the treatment modality, and the latter for evaluation of the patient's prognosis and the need for adjuvant therapy.

The UICC TNM classification for gastric cancer was renewed in 1997 and the 5th edition includes the number of metastatic lymph nodes: pN1 indicates metastases in 1 – 6, pN2 in 7 – 15, and pN3 in more than 15 regional lymph nodes (Sobin & Wittekind, 1997). The 5th edition classifies patients with T4N1-N3M0, T1-T3N3M0 or any T, any N and M1 tumours to stage IV (Sobin & Wittekind, 1997), compared to the 4th edition that classified patients with T4N2M0 or any T, any N and M1 tumours to stage IV (Hermanek & Sobin, 1987). The new staging is prognostically more accurate than the previous classification, which relied on the anatomic sites of metastatic lymph nodes (Fujii et al., 1999; Yoo et al., 1999; Katai et al., 2000; Celen et al., 2003). Survival among patients that have been classified into the same stage according to the new classification is more homogeneous. The depth of invasion and the new nodal staging have emerged as more significant prognostic factors than the previous nodal staging method (Yoo et al., 1999). The new UICC classification of nodal involvement has also been considered superior to the Japanese classification (Ichikura et al., 1999; Ichikawa et al., 2003).

Nodal staging according to the 5th edition (Sobin & Wittekind, 1997) classifies patients with more than 15 involved regional lymph nodes into a poor prognosis group (de Manzoni et al., 1999; Omejc et al., 2001). However, the requirement that at least 15 lymph nodes need to be examined, also in pT1 patients, has raised some concern (Nio et al., 2003) and prognostic accuracy might be improved by using the ratio of metastatic lymph nodes (number of metastatic lymph nodes/number of lymph nodes removed) (Kim et al., 1998; Kodera et al., 1998; Kunisaki et al., 2005). The current 6th edition was published in 2002 and includes some amendments concerning gastric

cancer, although the actual stage groups have not changed (Sobin & Wittekind, 2002). In the 6th edition, the T2 category is subdivided into T2a (tumour invades the muscularis propria) and T2b (tumour invades the subserosa). Adenocarcinomas of the gastroesophageal junction are also subdivided into "true" cardial or subcardial tumours.

Early gastric cancer is defined as gastric adenocarcinoma restricted to the gastric mucosa or submucosa, irrespective of regional lymph node involvement (Murakami et al., 1971). In a large retrospective review of 1,475 patients treated at the National Cancer Center in Tokyo, only 20 (1.4 %) died of recurrent disease (perioperative deaths and patients with noncurative operations excluded) (Sano et al., 1993). Five-year survival rates of over 90 % have consistently been reported for surgically treated early gastric cancer patients (Maehara et al., 1992; Folli et al., 1995; Yokota et al., 2000) and screening of asymptomatic patients has been instituted in Japan, where the disease is prevalent. Now early gastric cancer is diagnosed more often in Japan than in the Western countries (Everett & Axon, 1997). The prognosis is most favourable for patients with tumours confined to the mucosa and no lymph node metastases (Everett & Axon, 1997; Yokota et al., 2000).

Tumour size is not a part of the TNM stage classification for gastric cancer. Tumour size is usually a powerful predictor of prognosis by univariate survival analysis, but it does not seem to add prognostic information over and above the penetration depth and lymph node metastasis when data is analysed multivariately (Adachi et al., 1997; Yokota et al., 2002). Nevertheless, for treatment and prognosis the anatomic extent of the disease is not the only factor that needs to be considered (Gospodarowicz et al., 2001).

5.5.2 Tumour location

Cardia cancer has generally a worse prognosis than cancer of the distal parts of the stomach (Ito et al., 2004).

5.5.3 Age and gender

The incidence of gastric cancer increases with age. The mean age at diagnosis is 60 – 70 years and about 10 % of all gastric cancers are diagnosed in patients 40 years or

younger. In younger patients the diffuse type of carcinoma prevails (Kath et al., 2000; Kokkola & Sipponen, 2001). Prognosis does not seem to be affected by age, once survival data is adjusted for TNM stage (Blair & Schwarz, 2001; Kokkola & Sipponen, 2001; Kim et al., 2005a). Gastric cancer is more common among men than women (2:1) in elderly patients; this is a worldwide phenomenon which is independent of the incidence of gastric cancer (Kim et al., 2005a; Sipponen & Correa, 2002; Wang et al., 1996; Medina-Franco et al., 2000). This gender difference is not present among younger patients (Grabiec & Owen, 1985; Fujimoto et al., 1994; Sipponen & Correa, 2002; Kim et al., 2005a). The reasons for this age-related shift is not clear but could be related to a protective effect of oestrogen among premenopausal women, different initiation of *H. pylori* infection, or differences in chemical exposure during life (Ershler & Longo, 1997; Sipponen & Correa, 2002).

5.5.4 Borrmann's classification

Borrmann has classified the macroscopic appearance of gastric tumours into four types (Borrmann, 1926). Borrmann type I is characterised by protrusive tumours, type II by protrusive and ulcerating, type III by ulcero-infiltrative, and type IV by diffuse tumours (linitis plastica). Borrmann type IV cancer is associated with an unfavourable prognosis (Chen et al., 2002; Zhang et al., 2004).

5.5.5 Laurén's classification

Laurén's classification is the most common histological classification (Laurén, 1965). It distinguishes between two different histological types of gastric cancers and these types have different epidemiology, pathogenesis, genetic variation, and prognosis (Hermanek & Wittekind, 1995). The intestinal (well differentiated) type is characterized by large, distinct cells with large, irregular nuclei and the cells form glandular tubular-like structures. The diffuse (undifferentiated) type is more infiltrative, lacks the characteristic tubular gland-like structures and consists of small single cells in a non-organised pattern (Laurén, 1965).

Among younger patients, the incidence of the intestinal type of tumours has decreased along with the total incidence of gastric cancer and the diffuse type predominates (Laurén & Nevalainen, 1993). The total decline in gastric cancer incidence has been attributed to a decline in both intestinal and diffuse type of gastric cancer (Sipponen et

al., 1987; Lundegardh et al., 1991). The diffuse type is more common in gastric cancer of the cardia (Kim et al., 2005b) and has usually been reported to associate with a worse prognosis than the intestinal type (Yu et al., 1995; Hochwald et al., 2000).

5.5.6 Histological grade

The histological grade of the tumour refers to an evaluation of whether the malignant cells of the tumour represent a particularly aggressive form of gastric cancer or not. The grade is usually classified into four categories based on cell differentiation, number of mitoses per high power microscope field, adherence of the cells to original cells, homogeneity of the cellular pattern, and similarity of structures to the original organ. In gastric cancer, grade 1 tumours are well differentiated with similar characteristics as the original tissue, grade 2 are moderately well differentiated, grade 3 are poorly differentiated, and grade 4 are undifferentiated tumours, which cannot be recognised as having any characteristics of their tissue of origin. However, histological grade is subjective and cannot be determined quantitatively.

5.5.7 Tumour markers

A tumour marker is a substance whose concentration changes with malignancy in body fluids like blood, serum, urine, or peritoneal fluid, or in tissues of patients with cancer. A tumour marker may be produced by the tumour itself or by the body in response to the tumour. These markers can be used in diagnosis when a malignant tumour is suspected, although tumour markers are generally inadequate as screening tests. The diagnostic usefulness of tumour markers is determined by their sensitivity and specificity. Tumour markers are not unproblematic, since slightly altered concentrations are often found in benign conditions. Tumour markers may be useful for monitoring the effect or success of treatment and monitoring the course of disease once a particular tumour that affects the marker level has been diagnosed. The tumour marker concentration may also reflect the spread or the stage of the disease, be a sign of how cancer progress and may help determine the prognosis.

Advances in molecular biology have led to discoveries of new molecular markers of cancer tumourigenesis, angiogenesis, growth, invasion, and metastasis. An enormous effort is being put into studies on the use of oncogenes, tumour-suppressor genes, growth factors, growth factor receptors, adhesion molecules, and angiogenic factors as

new diagnostic biological markers, therapeutic targets, and prognostic factors of cancer (Houshmand & Zlotnic, 2003). Different techniques for identifying molecular changes in tumour tissue are available, e.g., polymerase chain reaction (PCR), DNA sequencing, immunoblotting, and immunohistochemistry. Immunohistochemistry is simple, fast, consistent with other methodologies, and can be applied to several, relatively small specimens at the same time, and is therefore a popular method for studying tumour markers. The actively dividing cancer cells also express large and chromatin-rich nuclei as a sign of an aberrant amount of chromosomes, which can be assessed by DNA flow cytometry.

Syndecan-1

Syndecan-1 (CD138; gene name SDC1; gene location 2p24.1) is a transmembrane heparan sulphate proteoglycan, one of a family of four, named syndecan 1 to 4 based on the order of cloning of their cDNAs (Bernfield et al., 1992). Syndecan-1 consists of a core protein with a transmembrane domain, a C-terminal cytoplasmic domain and an N-terminal extracellular domain, which attaches covalently long, unbranched carbohydrate polymers called glycosaminoglycans (GAGs) (Bernfield et al., 1992).

The variable chondroitin sulphate and heparan sulphate chains of the extracellular domain of syndecan-1 binds to different extracellular matrix components, such as collagen types I, III and V, fibronectin, thrombospondin, tenascin, amphoterin, and laminin (Koda et al., 1985; Bernfield & Sanderson, 1990; Salmivirta et al., 1991, Salmivirta et al., 1994). The extracellular domain of syndecan-1 stimulates actin polymerisation, participates in cell proliferation, suppresses malignant growth in different carcinoma cell lines, and regulates complex cell behaviour patterns, e.g., motility and invasiveness (Ridley et al., 1993; Liebersbach & Sanderson, 1994; Mali et al., 1994; Liu et al., 1998). Syndecan-1 may participate in signal transduction as a growth factor receptor, e.g., of the basic fibroblast growth factor (bFGF) (Elenius et al., 1990; Bernfield et al., 1992; Rapraeger, 1993). Syndecan-1 contacts the cytoskeleton through the cytoplasmic domain, and this mediates its organisation and influences cell shape (Elenius et al., 1990).

Syndecan-1 is expressed on all human cells in a way that is specific for cells, tissues and developmental stage (Kim et al., 1994; Salmivirta & Jalkanen, 1995). The

expression varies by maturation stage and follows morphogenetic rather than histological boundaries (Thesleff et al., 1988; Vainio et al., 1989). In adult tissues, syndecan-1 is predominantly expressed in the epithelium and this is most prominent in stratified squamous epithelia, e.g., the epidermis, the oral mucosa and the vaginal mucosa, and in keratinocytes (Hayashi et al., 1987; Inki et al., 1991). Syndecan-1 contributes to some regenerative processes, e.g., wound healing (Elenius et al., 1991; Elenius et al., 2004). Syndecan-1 is present at the basolateral surface of foveolar cells of the gastric mucosa, early in the scar tissue at the margins of healing gastric ulcers, and in the cells of intestinal metaplasia (Tanabe et al., 1999).

In mouse keratinocyte cell lines of different morphology and tumourigenicity the expression of syndecan was reduced in carcinoma cells compared to their normal counterparts (Inki et al., 1992). Loss of syndecan-1 is a characteristic feature of human hepatocellular carcinoma that has a high metastatic potential (Matsumoto et al., 1997). Loss of immunohistochemical expression of epithelial syndecan-1 correlates with a poor prognosis in patients with squamous cell carcinoma of the head and neck (Inki et al., 1994; Pulkkinen et al., 1997; Anttonen et al., 1999) and mesotheliomas (Kumar-Singh et al., 1998). Non-small-cell lung carcinomas, particularly poorly differentiated tumours with an invasive phenotype, express syndecan-1 poorly (Nackaerts et al., 1997), but the stroma of infiltrating ductal breast carcinomas have a strong expression (Stanley et al., 1999). Stromal syndecan-1 positivity could be a sign of aggressive malignant behaviour in breast tissue, since altered syndecan-1 expression, induction within the stroma and reduction or loss of expression the malignant cells themselves, could be critical for promoting the metastatic phenotype of infiltrating ductal mammary carcinoma.

p27

The 27-kDa-protein p27 (cyclin-dependent kinase inhibitor 1B; CDKN1B; KIP1; gene location 12p13.1) is a mitotic inhibitor of the cell cycle. It inhibits cyclin dependent kinase activity and interacts strongly with D-type cyclins in complex with cdk4 and – to a weaker extent – with cyclin E and cdk2 complexes (Toyoshima & Hunter, 1994). It functions as a negative regulator of G1 progression and could be a mediator of TGFβ induced G1 arrest (Toyoshima & Hunter, 1994). p27 could thus be a tumour suppressor – a notion that is supported by reports according to which cells lacking p27

expression do not enter a quiescent state (Toyoshima & Hunter, 1994). Phosphorylation and degradation via the ubiquitin-proteosome pathway are the main regulators of the expression of p27 (Pagano et al., 1995). Changed protein stability might be more important for the regulation of p27 levels than changed synthesis of the protein (Pagano et al., 1995). At any event, mutations are a rare cause for decreased p27 expression in cancer cell nuclei (Ponce-Castaneda et al., 1995). Immunohistochemistry seems therefore to be a suitable method for assessing the expression of p27 in neoplasias.

In healthy epithelial tissue the p27 protein is present in a large number of cells. The expression is clearly downregulated as a consequence of neoplastic transformation (Fernandez et al., 1999; Slingerland & Pagano, 2000; Zucchi et al., 2002). The expression of p27 in tumour cells of lymph node metastases is reduced in comparison with the primary gastric carcinoma (Kim et al., 2000). In gastric cancer, decreased expression of p27 correlates positively with tumour size, grade, lymphatic invasion, stage, invasion depth, number of lymph node metastases, and recurrence (Kim et al., 2000). Loss of p27 expression may be associated with a poor prognosis in patients with a variety of solid tumours, including breast (Leivonen et al., 2001), colon (Tenjo et al., 2000) and oesophagus cancer (Shamma et al., 2000). The results regarding gastric cancer are contradictory. Low expression of p27 has been reported to be an independent prognostic factor in several studies (Kwon et al., 1999; Ohtani et al., 1999; Sgambato et al., 2000), but not all (Feakins et al., 2000; Muller et al., 2000).

Tenascin-C

Tenascin-C (TNC; gene location 9q33) is a large glycoprotein of the extracellular matrix with a unique six-armed multidomain macromolecular structure (Schenk & Chiquet-Ehrismann, 1994). Other members of the non-collagenous extracellular matrix proteins are the laminins (Brown & Timpl, 1995) and fibronectins (Hynes, 1986). Extracellular matrix molecules are important for cell proliferation and morphogenesis – they guide migration, growth as well as differentiation of cells (Adams & Watt, 1993).

Tenascin-C is expressed in epithelial-mesenchymal interactions during a period of fetal morphogenesis (Aufderheide et al., 1987; Aufderheide & Ekblom, 1988; Erickson &

Bourdon, 1989; Natali et al., 1991) and again in adult tissues during wound repair (Mackie et al., 1988) and tumourigenesis (Erickson & Bourdon, 1989; Howeedy et al., 1990; Natali et al., 1991). Tenascin-C is expressed in the muscularis mucosae, muscularis propria and vessel walls of healthy gastric tissue, but not in healthy gastric mucosa or submucosal connective tissue (Ikeda et al., 1995).

The expression of tenascin-C seems to increase during tumourigenesis (Pilch et al., 1999). Cancer cells and adjacent stromal cells may produce tenascin, which seems to facilitate co-ordinated growth of the microenvironment surrounding tumours (Sakakura & Kusakabe, 1994; Ishihara et al., 1995). Local tumour invasion seems to be inhibited by stromal tenascin-C expression (Sugawara et al., 1991) and by covering the cancer nest tenascin-C might block cancer invasion. This implies that stromal tenascin-C expression could be associated with an improved prognosis (Sakakura & Kusakabe, 1994; Ishihara et al., 1995). In gastric cancer, tenascin is present in the fibrous stroma surrounding foci of cancer (Mackie et al., 1988; Ikeda et al, 1995).

Stromal tenascin-C expression has been associated with a better prognosis in cervical, colonic and breast cancer (Pilch et al., 1999; Sugawara et al., 1991; Shoji et al., 1993; Iskaros et al., 1997). In earlier studies of gastric cancer, stromal tenascin expression in the primary tumour has not been shown to correlate with prognosis (Ikeda et al., 1995; Ilunga & Iriyama, 1995; Zirbes et al., 1999).

TATI

The 6 kD tumour-associated trypsin inhibitor, TATI, (serine peptidase inhibitor, Kazal type 1; SPINK1; gene location 5q32) was first identified in the urine of a woman with ovarian cancer (Stenman et al., 1982). It was later found to be identical to the pancreatic secretory trypsin inhibitor PSTI (Huhtala et al., 1982). TATI is a strong inhibitor of trypsin, by which it is gradually degraded, and inhibits only slightly other serine proteinases (Fritz et al., 1967; Huhtala et al., 1982; Turpeinen et al., 1988). In the stomach its role is probably to prevent digestion of the gastric mucus (Freeman et al., 1990; Playford et al., 1991). In vitro, PSTI/TATI increases cell migration in wound repair and it may play a role in repairing tissue after tissue destruction (Marchbank et al., 1998). TATI might have an important function for maintaining and repairing tissue

and for protecting the mucous membrane against degradation. This raises the thought that TATI could also contribute to the inhibition of tumour spread.

Normally, TATI is expressed in the human pancreas, urinary tract, gall bladder, breast, kidney, fetal lung, and mucus-producing cells of the gastrointestinal tract, especially in the foveolar cells of the stomach (Fukayama et al., 1986; Haglund et al., 1986; Bohe et al., 1987; Freeman et al., 1990; Playford et al., 1994; Marchbank et al., 1996). TATI is also secreted into the gastric juice where it is stable under the neutral conditions of the protecting mucus layer (Freeman et al., 1990).

In cancers, TATI is often co-expressed with tumour-associated trypsin (Solakidi et al., 2003), suggesting that TATI plays the same role of protecting against tissue destruction in cancers as it does in the pancreas (Stenman, 1990; Stenman et al., 1991). TATI is expressed in several forms of cancer tissue, and increased levels of TATI in the serum have been reported in pancreatic, colorectal, gastric, lung, ovarian, renal cell, and bladder cancers (Haglund et al., 1986; Ogawa et al., 1987; Halila et al., 1988; Higashiyama et al., 1990; Loizate Toricaguena et al., 1991; Piantino & Arosaio, 1991; Järvisalo et al., 1993; Pasanen et al., 1995; Lukkonen et al., 1999; Paju et al., 2001; Kelloniemi et al., 2003). Of patients with gastric cancer 19 – 85 % have increased levels of TATI; the concentrations are related to TNM stage (Loizate Toricaguena et al., 1991; Piantino & Arosaio, 1991). For patients with ovarian, bladder and renal cell cancers, an increased serum level of TATI marks a poor prognosis (Venesmaa et al., 1994; Venesmaa et al., 1998; Paju et al., 2001; Kelloniemi et al., 2003). TATI/PSTI is expressed immunohistochemically in 83 % of gastric adenocarcinomas, but its prognostic value has not been previously assessed (Higashiyama et al., 1990).

p53

p53 is a 53-kD nuclear protein also known as tumour protein 53 (TP53; gene location 17p13.1). It consists of four domains: one that activates transcription factors, a core domain that recognises specific DNA sequences, a domain that is responsible for the tetramerisation of the protein, and a domain that recognises damaged DNA. p53 is a transcription factor that regulates the cell cycle, especially transition from G0 to G1 (Levine, 1997).

In non-neoplastic cells, p53 is continually produced and degraded and expression is low. The major regulator of p53 is Mdm2 (Moll & Petrenko, 2003). Expression of Mdm2 is, in turn, activated by p53 in an autoregulatory feedback loop. Binding of p53 by Mdm2 can trigger the degradation of p53 via the ubiquitin system and Mdm2 normally maintains p53 at a low level. DNA damage and other stress signals may activate protein kinases to phosphorylate p53, thereby increasing the p53 level and disrupt its binding with Mdm2 (Harris & Levine, 2005). Since Mdm2 expression is activated by p53, the increase of p53 increases concomitantly with Mdm2, which, however, has no biological effect as long as p53 is phosphorylated. After the DNA damage is repaired, p53 is quickly dephosphorylated and destroyed by the accumulated Mdm2. An increase of p53 proteins induces cell cycle arrest and this prevents replication of the damaged DNA and provides the cell with time for enzymatic repair of the DNA lesions by proteins, the transcription of which is activated by p53 (Kuerbitz et al., 1992). As a last resort, p53 can induce cell apoptosis (Yonish-Rouach et al., 1991).

If the p53 gene is damaged by mutagens (chemicals, radiation, or viruses), tumour suppression is severely reduced and this raises the probability that the cell will begin to divide unrestrictedly. Probably the most frequent tumourigenic mutations are those of the p53 tumour suppressor gene; more than 50 percent of all human tumours contain a mutation or deletion of the p53 gene (Hollstein et al., 1991). These mutations lead usually to loss of function of the p53 protein with failure to bind the consensus DNA binding site. The consequence of this is that DNA repair becomes defective, genetic disorders accumulate, cell ploidy becomes pathological and malignant transformation ensues (Kastan et al., 1991; Levine, 1997). People who inherit only one functional copy of p53 will most likely develop tumours in early adulthood, a disease known as the Li-Fraumeni syndrome. Immunohistochemically detected p53 is frequently (85 %) the mutant form, which is more stable than the wild-type p53 (Finlay et al., 1988) and does not generally induce Mdm2; this is why the p53 can accumulate at very high concentrations in the cell nucleus (Bodmer et al., 1992; Baas et al., 1994). The mutant form of the p53 protein can also inhibit normal p53 itself (Blagosklonny, 2002).

Mutated p53 occurs in several forms of cancer (Hollstein et al., 1991). In gastric cancer, immunohistochemical p53 expression has been reported in 13 – 64 % of

specimens (Seruca et al., 1992; Zafirellis et al., 2005). Overexpression of p53 may be related to a worse prognosis than normal expression in gastric cancer (Martin et al., 1992; Victorzon et al., 1996a; Lee et al., 2003; Fondevila et al., 2004) but this has not been a consistent finding (Motojima et al., 1994; Zafirellis et al., 2005).

p21

p21, also called cyclin-dependent kinase inhibitor 1A (CDKN1A, Cip1, Waf1; gene location 6p21.2) is a transcriptional target of p53 by which it is activated (el-Deiry et al., 1993). However, there are also p53 independent pathways of p21 induction (Elbendary et al., 1994). p21 inhibits the phosphorylation of cyclin-cdk2 or cyclin-cdk4 complexes and induces cell cycle arrest at the G1/S checkpoint (Sheikh et al., 1995). Evidence is increasing that p21 is also a major inhibitor of p53-induced as well as p53-independent apoptosis, but why this is the case is unknown (Cayrol et al., 1998; Gartel & Tyner, 2002).

p21 protein is produced in normal gastric mucosa and is lost in gastric cancer (Xie et al., 2004). There are no known mutations of the p21 gene (Park et al., 1998). The role of p21 for survival prediction is still unclear (Tsihlias et al., 1999). In gastric cancer, patients that lack p21 expression in the tumour may have a favourable prognosis (Okuyama et al., 2002) but, again, this has not been the case in all studies (Al-Moundhri et al., 2005).

bcl-2

The bcl-2 protein, coded by the bcl-2 proto-oncogene (gene location 18q21.33), is a mitochondrial protein integrated in the outer mitochondrial membrane. bcl-2 is closely linked to p53 for apoptosis regulation by inhibiting p53 induced apoptosis (Yonish-Rouach et al., 1991; Bissonnette et al., 1992; Korsmeyer et al., 1992; Oren et al., 1992).

Overexpression of bcl-2 is an early event in gastric tumourigenesis. It takes place before gastric dysplastic changes are seen (Anagnostopoulos et al., 2005). Overexpression of bcl-2 is also linked to suppression of cellular proliferation and to less aggressive forms of tumours (Saegusa et al., 1995). The prognostic significance of bcl-2 in gastric cancer is still contradictory, as some studies report a favourable

prognosis in patients with bcl-2 positive tumours (Inada et al., 1998) and others do not (Muller et al., 1998).

5.5.8 DNA ploidy and S-phase fraction (SPF)

Healthy human cells have 23 pairs of chromosomes, i.e., a total of 46, and are called diploid. A disturbed number of chromosomes or chromosome sets is called aneuploidy. Aneuploidy is a good marker of a malignant cell population and an abnormal amount of DNA in solid tumours is a specific sign of neoplasia (Barlogie et al., 1980; Barlogie et al., 1983). DNA flow cytometry measures the DNA amount of single cells as they flow past a laser light source that emits coherent light at a specific wavelength. The DNA, which has been stained with a fluorescent dye, is exited by the laser beam and emits light at a longer wavelength. The emitted light is picked up by detectors and the signal is digitally analysed. The technique to analyse the DNA amount from single cells in paraffin embedded tumour tissues was describe by Hedley et al. (Hedley et al., 1983).

Several studies have reported that aneuploidy is a sign of a poor prognosis in gastric cancer (Setälä et al., 1997; Lanza et al., 1998; Pinto et al., 1999; Danesi et al., 2000; Russo et al., 2001; Michels et al., 2004). The S-phase fraction (SPF) is a measure of the percentage of cells in the DNA synthesis phase of the cell cycle. The S-phase fraction can be used together with the proliferative DNA index to give a more complete understanding of how aggressively a tumour might be growing. A high SPF is apparently a sign of poor prognosis of gastric cancer patients (Ohyama et al., 1990; Setälä et al., 1998; Russo et al., 2001).

6. AIMS OF THE STUDY

The prognostic evaluation of gastric cancer patients is usually based on the TNM-stage classification by the UICC. There is a need for more accurate prognostication as treatments develop. Strong prognostic factors facilitate an individualised evaluation of prognosis and make it easier to compare treatment results between centres.

The specific aims of this study were to assess:

1. the immunohistochemical expression and prognostic significance of the proteoglycan syndecan-1 in tumours from patients with primary gastric cancer and its association with known prognostic factors

2. the immunohistochemical expression of the cell-cycle protein p27 in gastric cancer tissue and its association with prognosis and other clinicopathological factors

3. the stromal staining pattern of the extracellular matrix protein tenascin-C and to evaluate its prognostic significance and association with other prognostic variables of patients with gastric cancer

4. the immunohistochemical expression of the enzyme inhibitor TATI in gastric cancer tissue and to assess the association of TATI with patient survival and other prognostic factors

5. the prognostic value of the known markers for apoptosis, p53, p21 and bcl-2, and of DNA ploidy and SPF in patients with gastric cancer. SPF and DNA ploidy were measured by flow cytometry.

6. all factors studied by multivariate survival analysis to identify novel independent prognostic variables representing different aspects of tumour biology in gastric cancer.

7. PATIENTS, MATERIALS, AND METHODS

7.1 Patients (I-V)

Clinical data were collected from the medical records of 362 patients with gastric cancer treated at the Second Department of Surgery, Helsinki University Central Hospital between 1983 and 1999. The data included age at diagnosis, gender, Borrmann's classification, Laurén's classification, histological grade when available, depth of tumour penetration (pT), extent of spread to regional lymph nodes (pN), the presence or absence of distant metastases (pM), TNM-stage classification, tumour size, tumour location, type of surgery, estimated cure by surgery, and clinical outcome. Of the patients, 342 underwent surgery for histologically verified gastric adenocarcinoma. Four patients were excluded due to lack of clinical data. Archival tissue specimens were available of 337 of the patients. TNM-staging was performed according to the 4th edition UICC staging procedures (Hermanek & Sobin, 1987). There were 52 (15 %) stage IA, 48 (14 %) stage IB, 41 (12 %) stage II, 67 (20 %) stage IIIA, 29 (9 %) stage IIIB, and 100 (30 %) stage IV patients. Total or partial gastrectomy with extended (D2-D4) lymphadenectomy was performed in 34 patients (10 %), total gastrectomy and lymphadenectomy (D1) in 161 (48 %), subtotal gastrectomy and lymphadenectomy (D1) in 58 (17 %), and partial gastrectomy and lymphadenectomy (D1) in 84 (25 %). Of the patients, 176 (52 %) were operated on with curative intent. Of the 84 undergoing partial gastrectomy, 27 (32 %) were operated on with curative intent. During surgery, 280 (83 %) of the tumours were classified macroscopically into the four types of Borrmann (Borrmann, 1926): polypoid (type I), ulcero-fungating (type II), ulcero-infiltrative (type III) and diffuse (type IV). Six of the patients had adenocarcinoma in the gastric stump after previous partial gastrectomy for non-malignant disease. Survival data was obtained from patient records and Statistics Finland. The survival data was checked in January 2004. The median follow-up time for patients alive at the end of follow-up was 12.5 years (range 4.7 – 20.8). The clinicopathological characteristics of the patients are presented in Table 1.

Table 1. Clinicopathological characteristics of 337 patients with gastric cancer.

Clinicopathological variable	Patients
Age	
< 66 years	165
≥ 66 years	172
Gender	
Female	163
Male	174
Stage	
Stage IA	52
Stage IB	48
Stage II	41
Stage IIIA	67
Stage IIIB	29
Stage IV	100
Penetration depth	
Mucosa (T1)	26
Submucosa (T1)	33
Muscularis propria (T2)	48
Subserosa (T2)	12
Serosa (T3)	155
Adjacent structures (T4)	63
Lymph node metastases	
N0	152
N1	95
N2	89
Not available	1
Distant metastases	
M0	244
M1	93
Tumour location	
Upper 1/3	69
Middle 1/3	114
Lower 1/3	126
Diffuse	20
Stump	6
Not available	2
Laurén's classification	
Intestinal	142
Diffuse	195
Borrmann's classification	
Type I	51
Type II	92
Type III	80
Type IV	58
Not available	55
Tumour size median	
≤ 5 cm	185
> 5 cm	146
Not available	6
Resectability	
Intent to cure	176
Non-curative	143
Not available	18
Histological grade	
Grade I	18
Grade II	39
Grade III	59
Grade IV	6
Not available	215

7.2 Tissue samples (I-V)

Tissue samples obtained at surgery were fixed in 4 % buffered formaldehyde for 12 to 48 h, processed, embedded in paraffin, and stored in the files of the Department of Pathology, University of Helsinki. An experienced pathologist reviewed all haematoxylin and eosin or Alcian blue periodic acid-Schiff (AB-PAS) stainings, with the most representative samples chosen for further analysis. Histological classification was assessed according to Laurén's criteria into either intestinal (n = 142) or diffuse (n = 195) type (Laurén, 1965) (Table 1).

7.3 Methods

7.3.1 Immunohistochemistry (I-V)

For immunohistochemistry, 4-micrometer thick, freshly cut sections of the paraffin-embedded samples were fixed on pre-treated Super Frost® Plus (Menzer-Gläser, Germany) slides and dried for 12 to 24 h at 37°C. Sections were deparaffinised in xylene and rehydrated through graded concentrations of ethanol to distilled water. Deparaffinised tissue sections were pre-treated in 0.5 % trypsin (Difco Laboratories, Detroit, MI, USA) in PBS solution for 30 min at 37°C for tenascin-C, TATI, p21, and bcl-2 or in a 700 W microwave oven for 4 x 5 min in an 0.3 % citrate acid buffer, at pH 6.0, cooled for 20 min in room temperature and washed in 1:10 phosphate buffered saline (PBS):distilled water solution for syndecan-1, p27, and p53 antigen retrieval. The sections were incubated in 0.3 % hydrogen peroxide in methanol for 30 min to quench endogenous peroxidase activity and incubated in a blocking solution consisting of non-immune horse serum, diluted 1:67 for 15 min to block non-specific binding sites. The primary antibodies were applied overnight in PBS containing 0.1 % sodium azide (Merck Ltd., Poole, UK) and 0.5 % bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA) at room temperature. The primary antibodies were all monoclonal (MAb) and are listed in Table 2. For intensification of the primary antigen signals of p21 and bcl-2, sections were treated with biotinyl tyramide and streptavidin conjugated to horseradish peroxidase (Catalyzed Signal Amplification, K1500, Dako, Glostrup, Denmark) following the manufacturer's instructions. Bound antibody was visualised by the avidin-biotin immunoperoxidase technique (ABC) (Vectastain ABComplex, Vector Laboratories, Burlingame, CA, USA) following the manufacturer's instructions. Sections were incubated with biotinylated second-layer antibody (1:200; Vector Laboratories Inc., Burlingame, CA, USA) and peroxidase-

labelled ABC for 30 min each. All dilutions were made in PBS (pH 7.2) and all incubations in the ABC method were carried out in humid chambers at room temperature. Between each step in the staining procedure, the slides were rinsed in three changes of PBS.

Table 2. Details on immunohistochemistry and cut-off values of different tumour markers (I-V).

Tumour marker	Antibody clone	Dilution	Cut-off for positivity
SDC1 (I)	B-B4, Serotec, Oxford, UK	1:100	> 60 % (epithelial) stromal staining
p27 (II)	57; Transduction Laboratories, Lexington, KY, USA	1:500	>5%
TNC (III)	DB7, Biohit Diagnostics, Helsinki, Finland	1:2000	strong stromal
TATI (IV)	EC8, in-house, Finland (Osman et al., 1993)	0.2 μg/ml	> 50%
p53 (V)	DO7, Dako, Glostrup, Denmark	1:300	> 20%
p21 (V)	4D10, Novocastra Laboratories, Ltd., Newcastle upon Tyne, UK	1:20	> 20%
bcl-2 (V)	124, Dako, Glostrup, Denmark	1:20	> 20%

Abbreviations: SDC1, syndecan-1; TNC, tenascin-C; TATI, tumour-associated trypsin inhibitor.

The cut-off levels were selected as follows:

- Loss of epithelial syndecan-1 (≤ 60 % of cancer cells positive) was considered as an alteration due to malignancy because syndecan-1 is normally expressed in gastric mucosa.
- Stromal syndecan-1 was considered either positive or negative.
- A tumour was considered p27negative if ≤ 5 % of the cancer cell nuclei expressed p27.
- Strong stromal tenascin-C immunoreactivity was considered positive as this was the cut-off that resulted in the highest significance according to cancer specific survival.
- For TATI there was a cut-off between high (> 50 %) and lower (≤ 50 %) TATI expression between which survival clearly differed.
- For p53, p21 and bcl-2 expression, a cut-off of 20 % (commonly used in the literature) was chosen.

The peroxidase staining was visualised with 3-amino-9-ethylcarbazole (A-5754; Sigma Chemical Co., St. Louis, MO, USA), 0.2 mg/ml in 0.05 M acetate buffer, pH 5.0, containing 0.03 % perhydrol at room temperature for 15 min. Finally, sections were lightly counterstained in Mayer's haematoxylin, cleared in tap water and mounted in

an aqueous mounting medium (Aquamount; BDH, Poole, UK). The procedure is schematically presented in Figure 2.

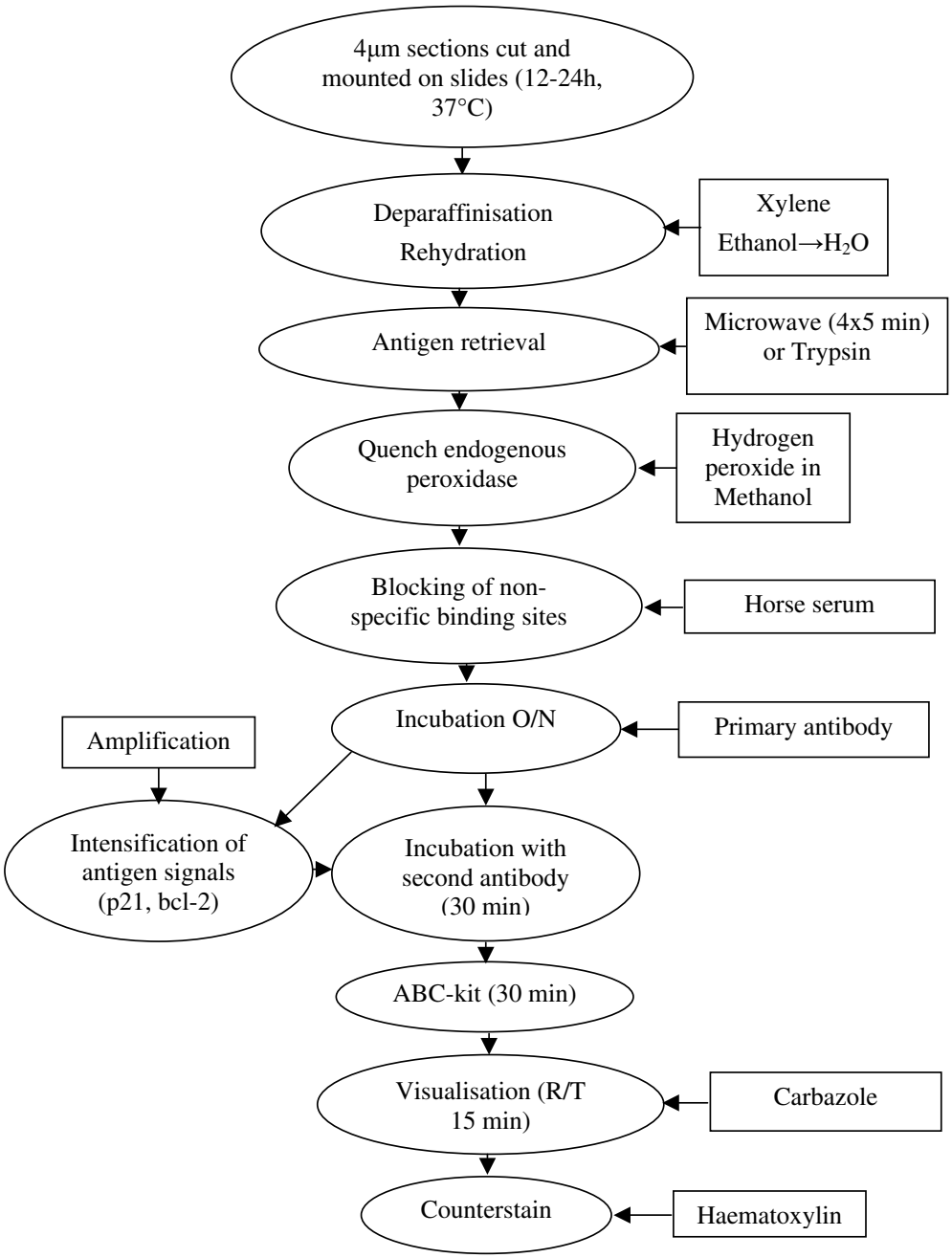

Figure 2. Overview of immunohistochemical staining procedure. Abbreviations: O/N, overnight; R/T, room temperature.

7.3.2 DNA flow cytometry (V)

For DNA flow cytometry a modification of the method of Hedley et al. (Hedley et al., 1983) was applied (Victorzon et al., 1996b). Two 50-µm sections were treated with 0.4 mg/ml proteinase K (Sigma Chemical Co., St. Louis, MO, USA) for 30 min at room temperature. After filtration, the nuclei were treated with 100 µg/ml ribonuclease to remove double-stranded RNA and stained with 50 µg/ml ethidium bromide (Sigma Chemical Co., St. Louis, MO, USA) for at least 1 h. The DNA content was determined by flow cytometric analysis (FACScan, Becton Dickinson, Mountain View, Calif., USA) using 15 mW excitation at 488 nm, and the total emission between 562 and 607 nm was recorded. The lowest peak was assigned a DNA index (DI) value of 1.00, and DI values of other peaks were based on this reference; no standard DI value was used as the staining intensity of fixed nuclei varies between samples. Possible hypodiploid peaks were identified as diploid and the normal diploid peak as hyperdiploid. If the DI was 1.20 or less, i.e., near diploid aneuploid, the sample was considered diploid. The S-phase fraction (SPF) was calculated in 274/314 (87 %) of the tumours using the Cellfit program of the FACScan flow cytometer or, if there was an abnormal skewness of the G1 peak to the right, manually by a modified rectilinear method; the lower SPF was chosen. If the sample contained less than 15 % aneuploid cells, the SPF was not calculated. At least 10,000 nuclei from each specimen were analysed. The histograms were analysed by a pathologist unaware of the patients' clinical outcome.

The number of patients and successful staining or flow cytometric results in each study is given in Table 3.

Table 3. Number of patients and immunohistochemical analysis by study.

	Study	**I**	**II**	**III**	**IV**	**V**
Marker n	Time	1983-1996	1983-1996	1983-1996	1983-1999	1983-1999
SDC1		296				
p27			316			
TNC				314		
TATI					336	
p53						336
p21						317
bcl-2						315
DNA pl						306
SPF						278

Abbreviations: SDC1, syndecan-1; TNC, tenascin-C; TATI, tumour-associated trypsin inhibitor; DNA pl, DNA ploidy; SPF, S-phase fraction.

7.3.3 Statistical analyses (I-V)

The χ^2 test was used to test for associations between categorical factors (I-V), and Spearman's rank correlation (r_s) (V) to test for correlations between continuous parameters. Bonferroni's adjustment of the p-value was performed in article V, where several variables were compared. Life tables were calculated according to the Kaplan-Meier method (I-V). Gastric cancer specific survival was calculated from the date of diagnosis to date of death due to gastric cancer. Death of intercurrent causes, patients alive at the end of follow-up, and patients lost to follow-up were censored. The significance of differences in survival between groups was calculated by the logrank test, or logrank test for trend if three or more ordered categories were compared (I-V). Multivariate survival analysis was performed by backward stepwise Cox proportional hazards regression (I-V). A p-value of 0.05 was adopted as the limit for inclusion of a variable. The StatView 5.0 software for Windows (SAS Institute Inc., Cary, NC, USA) and STATA 9.0 (Stata CorpLP, TX, USA) were used for statistical analysis.

8. RESULTS

8.1 Survival

8.1.1 Gastric cancer specific survival

Figure 3 shows the gastric cancer specific survival curve of the entire series of 337 patients with gastric cancer and Figure 4 the survival according to the TNM stage. The gastric cancer specific 5-year survival was 35.1 % (CI 95 % = 29.8 - 40.4) and the median survival time 2.0 years. During follow-up, 214 (64 %) of the patients died of gastric cancer; the last observed death due to gastric cancer was at 7.75 years from diagnosis.

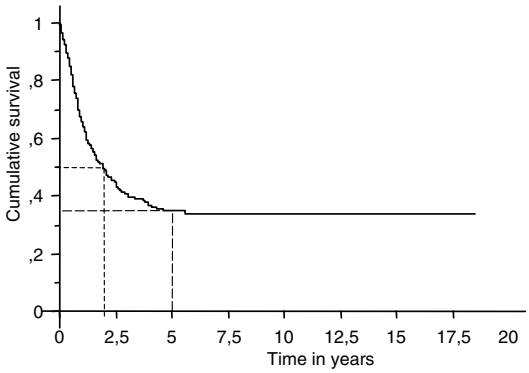

Figure 3. Cumulative disease specific survival of 337 patients with gastric cancer.

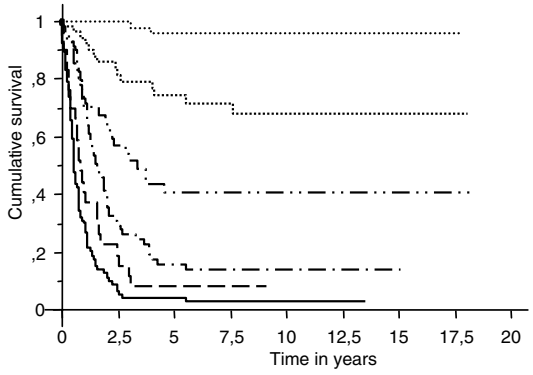

Figure 4. Gastric cancer specific survival by TNM stage of 337 patients with gastric cancer. Stage IA (····· , n = 52), stage IB (····, n = 48), stage II (—·· —, n = 41), stage IIIA (—·— , n = 67), stage IIIB (—— ;n = 29), stage IV (—— ;n = 100) (p < 0.0001).

8.1.2 Survival by clinicopathological variables

Univariate survival analysis of the clinicopathological variables is shown in Table 4. Histological grade was analysed only in the first studies because the number of patients in whom grade was assessed was incomplete. Grade was not routinely recorded.

Table 4. Univariate analysis of the relationship between preoperative characteristics and survival in 337 patients with gastric cancer.

Clinicopathological variable	Patients	Cumulative 5-year survival %	95 % CI	χ^2	p-value
Age				10.37	0.0013
< 66 years	165	44	36 - 51		
≥ 66 years	172	26	19 - 33		
Gender				1.42	0.23
Female	163	38	31 - 46		
Male	174	32	25 - 39		
TNM stage*				224.83	<0.0001
Stage IA	52	96	90 - 100		
Stage IB	48	74	61 - 87		
Stage II	41	40	24 - 56		
Stage IIIA	67	15	6 - 24		
Stage IIIB	29	7	0 - 17		
Stage IV	100	3	0 - 7		
Penetration depth*				130.26	<0.0001
Mucosa (T1)	26	96	88 - 100		
Submucosa (T1)	33	78	64 - 93		
Muscularis propria (T2)	48	62	48 - 77		
Subserosa (T2)	12	58	30 - 86		
Serosa (T3)	155	18	12 - 24		
Adjacent structures (T4)	63	2	0 - 6		
Lymph node metastases*				178.96	<0.0001
N0	152	67	59 - 74		
N1	95	14	7 - 22		
N2	89	4	0 - 8		
Not available	1				
Distant metastases				137.48	<0.0001
M0	244	47	40 - 53		
M1	93	4	0 - 8		
Tumour location				31.00	<0.0001
Upper 1/3	69	22	12 - 32		
Middle 1/3	114	49	40 - 59		
Lower 1/3	126	37	28 - 46		
Diffuse	20	0	0		
Stump	6	0	0		
Not available	2				
Laurén's classification				0.01	0.96
Intestinal type	142	34	26 - 42		
Diffuse type	195	36	29 - 43		
Borrmann's classification				26.06	<0.0001
Type I	51	30	17 - 43		
Type II	92	50	39 - 61		
Type III	80	25	16 - 35		
Type IV	58	17	7 - 27		
Not available	56				
Tumour size median				67.17	<0.0001
≤ 5 cm	185	53	46 - 60		
> 5 cm	146	12	7 - 18		
Not available	6				
Histological grade*				4.09	0.043
Grade I	19	27	6 - 49		
Grade II	42	26	12 - 40		
Grade III	64	21	11 - 31		
Grade IV	7	0	0		
Not available	205				
Resectability				195.64	<0.0001
Intent to cure	176	61	54 - 69		
Non-curative	143	3	0 - 6		
Not available	18				

* ; χ^2 and corresponding p-values were calculated using the logrank test for trend, other corresponding p-values were calculated using the logrank test.
Abbreviation: 95 % CI = 95 % confidence interval

8.2 Tumour markers

8.2.1 Syndecan-1 (I)

Epithelial syndecan-1 immunoreactivity

High epithelial syndecan-1 immunoreactivity (> 60 % positive cancer cells) was seen in 99 of 296 (33 %) tumours (Table 5). Corresponding to a loss of syndecan-1 immunoreactivity, moderate staining (21 – 60 %) was seen in 71 (24 %), weak staining (5 – 20 %) in 64 (22 %), and no staining (<5 %) in 62 tumours (21 %).

Loss of syndecan-1 immunoreactivity (≤ 60 % of the cancer cells positive) was significantly associated with positive stromal syndecan-1 staining ($p = 0.002$), a higher clinical TNM stage (stage II – IV) ($p = 0.001$), deep penetration of the primary tumour (subserosa or deeper) ($p = 0.002$), regional lymph node metastases (N1 or N2) ($p = 0.001$), a tumour size larger than the median (> 5 cm) ($p = 0.003$), location of the primary tumour in the upper third of the stomach ($p = 0.001$), and intestinal type of tumour according to Laurén's classification ($p = 0.008$). There was no significant association between syndecan-1 immunoreactivity and age ($p = 0.67$), gender ($p = 0.30$), distant metastases ($p = 0.26$), grade of differentiation ($p = 0.23$), or Borrmann's classification ($p = 0.24$).

The 5-year cumulative survival of patients with high epithelial syndecan-1 expression was 47 % as compared to 26 % for patients with loss of syndecan-1 expression (moderate, weak and negative) ($p = 0.001$; Table 5). The median survival times of the patients with high, moderate, weak, and no epithelial syndecan-1 expression were 3.5, 2.1, 2.1, and 2.0 years, respectively ($p = 0.002$; logrank for trend). The differences in survival of patients with negative, weak or moderate epithelial syndecan-1 expression was statistically not significant ($p = 0.52$).

Stromal syndecan-1 immunoreactivity

There was stromal syndecan-1 immunoreactivity in 28 of 296 (9 %) tumours (Table 5). Positive syndecan-1 stromal expression correlated with Borrmann's type I cancer ($p = 0.001$), loss of (≤ 60 %) epithelial syndecan-1 expression ($p = 0.002$), and the intestinal type of cancer ($p = 0.004$). Significant associations were not observed between stromal syndecan-1 immunoreactivity and age ($p = 0.38$), gender ($p = 0.13$), stage of disease (p

Table 5. Univariate survival analysis of the series of tumour markers and flow cytometric characteristics (I-V).

Tumour marker	Patients n (%)	5-year survival %	χ^2	p-value
Syndecan-1, epithelial			10.53	0.001
Low (≤ 60 %)	197 (67)	26		
High (> 60 %)	99 (33)	47		
Syndecan-1, stromal			5.48	0.019
Negative	268 (91)	34		
Positive	28 (9)	16		
p27			0.32	0.57
Negative (≤ 5 %)	241 (76)	32		
Positive (> 5 %)	75 (24)	36		
Tenascin-C, stromal			7.79	0.005
Low	192 (61)	26		
Strong	122 (39)	42		
TATI			7.62	0.006
Low (≤ 50 %)	183 (54)	28		
High (> 50 %)	153 (46)	43		
p53			10.30	0.001
Low (≤ 20 %)	232 (69)	40		
High (> 20 %)	104 (31)	24		
p21			7.46	0.006
Low (≤ 20 %)	274 (86)	33		
High (> 20 %)	43 (14)	20		
bcl-2			2.50	0.11
Low (≤ 20 %)	291 (92)	32		
High (> 20 %)	24 (8)	13		
DNA Ploidy			29.89	< 0.0001
Diploid	223 (73)	39		
Aneuploid	83 (27)	13		
SPF			24.18	< 0.0001
Low (< 7.6 %)	141 (51)	46		
High (≥ 7.6 %)	137 (49)	19		

Abbreviations: TATI, tumour-associated trypsin inhibitor; SPF, S-phase fraction; 95 % CI, 95 % confidence interval.

= 0.34), penetration depth (p = 0.27), regional lymph node metastases (p = 0.55), distant metastases (p = 0.89), tumour size (p = 0.76), or tumour location (p = 0.16).

Patients with stromal syndecan-1 immunoreactivity had a significantly more unfavourable outcome compared to patients with no stromal staining, with 5-year cumulative survival rates of 16 % and 34 %, respectively (p = 0.019; Table 5).

By multivariate survival analysis, stromal syndecan-1 immunoreactivity turned out to be an independent prognostic factor, in addition to TNM stage, estimated cure by surgery, and tumour size. Epithelial syndecan-1 immunoreactivity, regional nodal, and distant metastases, histological type, age, gender, Borrmann's class, grade, and tumour location did not add significant prognostic information. Entering the epithelial syndecan-1 score levels as a continuous variable did not affect the results, either.

8.2.2 p27 (II)

p27 immunoreactivity was very weak or absent (≤ 5 % of the cancer cell nuclei positive) in 241 of 316 (76 %) tumours (Table 5), while 301 (95 %) expressed p27 in less than 50 % of cancer cell nuclei. There was no significant association between p27 and the clinicopathological variables; age (p = 0.65), gender (p = 0.78), stage (p = 0.24), regional nodal metastases (p = 0.74), distant metastases (p = 0.67), penetration depth (p = 0.70), tumour location (p = 0.67), Laurén's class (p = 0.08), Borrmann's class (p = 0.33), tumour size (p = 0.82), grade of differentiation (p = 0.11), or estimated cure by surgery (p = 0.72).

In survival analysis, there was no significant difference between patients with p27 negative (≤ 5 %) and positive (> 5 %) tumours; the cumulative gastric cancer specific 5-year survival was 32 % and 36 %, respectively (p = 0.57; Table 5).

By multivariate survival analysis stratified by estimated cure by surgery, stage of disease was the only independent prognostic factor (p < 0.001). p27 did not add significant prognostic information.

8.2.3 Tenascin-C (III)

Tenascin-C immunoreactivity was present within the tumour stroma in 297 of 314 (95 %) cases. Strong tenascin-C expression was seen in 122 (39 %) of the tumour stromas (Table 5), 121 (39 %) stained moderately positive, 54 (17 %) had occasional weak staining, and 17 (5 %) tumours were negative for tenascin-C.

There was a statistically significant correlation between strong tenascin-C expression and low stage ($p = 0.002$), superficial tumour penetration ($p = 0.02$), tumour location at the distal third of the stomach ($p = 0.03$), and estimated cure by surgery ($p = 0.008$). There was no statistically significant correlation between tenascin-C expression and nodal status, distant metastases, age, Laurén's classification, gender, median tumour size, or Borrmann's classification.

The cumulative gastric cancer specific 5-year survival of patients with strong tenascin-C expression in tumour stroma was 42 % (95 % CI = 40 – 44 %), compared to 30 % (95 % CI = 28 – 33 %) in those with moderate, 20 % (95 % CI = 17 – 23 %) in those with occasional and 14 % (95 %CI = 9 – 18 %) in those with negative tenascin-C staining ($p = 0.006$, logrank for trend) (Figure 5), respectively. Patients with strong stromal tenascin-C expression were compared to the combined group of patients with moderate, weak, and negative staining. This was found to be the cut-off that resulted in the highest significance according to cancer specific survival between tenascin-C expression groups ($p = 0.005$; Table 5; Figure 5).

Tenascin-C did not add significant prognostic information after adjustment for TNM stage, when analysed by multivariate survival analysis stratified by estimated cure by surgery.

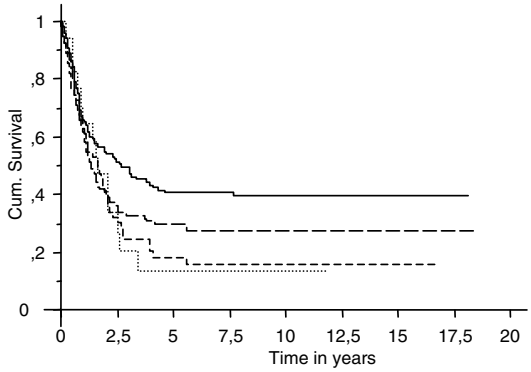

Figure 5. Gastric cancer specific survival according to strong (— ;n = 122) , moderate (– –;n = 121), weak (- - ; n = 54), and negative (····· , n = 17) stromal tenascin-C staining (p = 0.006).

8.2.4 TATI (IV)

TATI immunoreactivity in cancer cells was high in 153 of 336 (46 %) primary tumours (Table 5), moderate in 56 (17 %), weak in 56 (17 %), and absent in 71 (21 %).

There was a significant association between high TATI expression and low stage (p = 0.007), superficial tumours (p = 0.005), absence of nodal metastases (p = 0.015), absence of distant metastases (p = 0.022), estimated cure by surgery (p = 0.012), and epithelial syndecan-1 expression (p = 0.036). There was no significant association between the expression of TATI and age, gender, tumour location, Laurén's class, Borrmann's class, tumour size, stromal syndecan-1, tenascin-C, or p27 expression.

The 5-year survival of patients with high TATI expression in the primary tumour was 43 %, compared to 31 % for patients with moderate, 18 % for patients with weak, and 34 % for patients with negative TATI expression (p = 0.015, Figure 6). The 5-year survival of patients with high TATI expression (43 %) was significantly longer when compared to the combined group of patients with moderate, weak and negative expression who had a 5-year survival of 28 % (p = 0.006; Table 5). There was no significant difference in survival between patients with moderate, weak or negative TATI expression (p = 0.29).

TATI did not add prognostic information after adjustment for TNM stage (p < 0.001) and age at the time of diagnosis (p = 0.022), when analysed by multivariate survival analysis stratified for estimated cure by surgery.

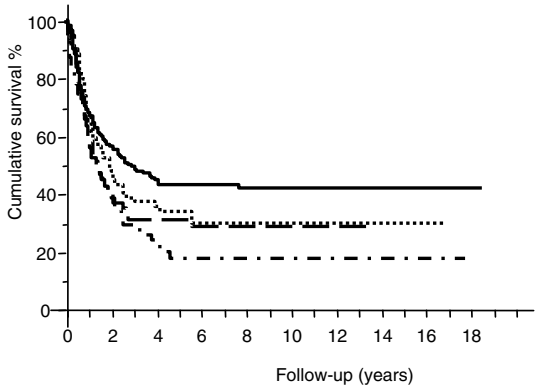

Figure 6. Gastric cancer specific survival according to high (— , n = 153), moderate (— — , n = 56), weak (— · — , n = 56), and negative (····· , n = 71) TATI immunoreactivity (p = 0.015).

8.2.5 p53 (V)

p53 immunoreactivity in cancer cell nuclei was observed in 131 of 336 (39 %) tumours, whereas 205 (61 %) tumours were p53 negative. High p53 expression (> 20 % of the cancer cell nuclei positive) appeared in 104 of 336 (31 %) tumours (Table 5). There were significant correlations after Bonferroni's correction at an α-value of 0.00032 between p53 and SPF (r_s = 0.38, $p < 0.0001$) and between p53 and p21 (r_s = 0.22, $p < 0.0001$). High p53 expression was associated with aneuploid tumours (p = 0.0002). The intestinal type of tumours had high p53 expression more often than the diffuse type of tumours (p = 0.002).

Patients with high (> 20 %) p53 expression had significantly shorter gastric cancer specific survival than patients with weak (≤ 20 %) p53 expression; the median survival times were 12.7 and 24.5 months, respectively (p = 0.004) and 5-year survivals 24 % and 40 % (p = 0.001; Table 5).

8.2.6 p21 (V)

High p21 expression (> 20 % positive cancer cell nuclei) was seen in 43 of 317 (14 %) tumours. p21 was absent in 231 (73 %) of the tumours (Table 5). There was a significant correlation after the Bonferroni's correction between p21 and p53 (r_s = 0.22, $p < 0.0001$).

The prognosis of patients with a high p21 tumour expression was significantly worse compared to low expression: the median survival times were 10.5 and 19.9 months, respectively ($p = 0.007$) and the 5-year survival 20 % and 33 % ($p = 0.006$; Table 5).

8.2.7 bcl-2 (V)

High bcl-2 expression (> 20 % positive cancer cells) was observed in 24 of 315 (8 %) tumours while 291 (92 %) were negative for bcl-2 (Table 5). After Bonferroni's correction, high bcl-2 associated with positive p27 expression ($p < 0.0001$).

There was no significant difference in survival between patients with expression of bcl-2 and a lack of expression of bcl-2 ($p = 0.11$; Table 5).

8.2.8 DNA ploidy (V)

The DNA index (DI) could not be estimated in 7 (2 %) of the 313 tumours because of background debris or a low number of intact cells. The median coefficient of variation (CV) was 6.9 %. Of the 306 tumours, 83 (27 %) were aneuploid and 223 (73 %) diploid (Table 5). The 7 near-diploid tumours (DI 1.0 – 1.2) were analysed as diploid. Aneuploidy was associated with high TNM stage ($p < 0.0001$), high p53 expression ($p = 0.0002$), intestinal type of tumours according to Laurén ($p < 0.0001$), high SPF ($p < 0.0001$), and high epithelial syndecan-1 (>60 %) expression ($p = 0.0003$).

The survival time was significantly shorter in patients with aneuploid than diploid tumours: the 5-year survivals were 13 % and 39 %, respectively ($p < 0.0001$; Table 5; Figure 7a). This was especially evident in the pT1-2 subgroup, where the 5-year survivals were 13 % and 82 %, respectively ($p < 0.0001$; Figure 7b).

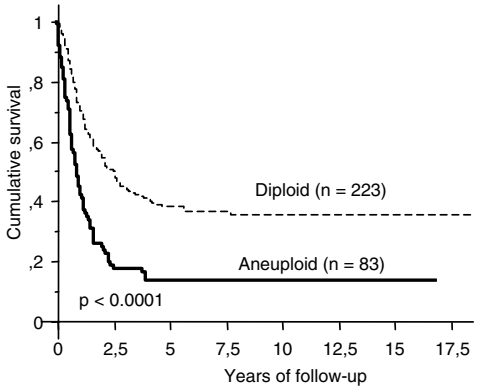

Fig. 7a

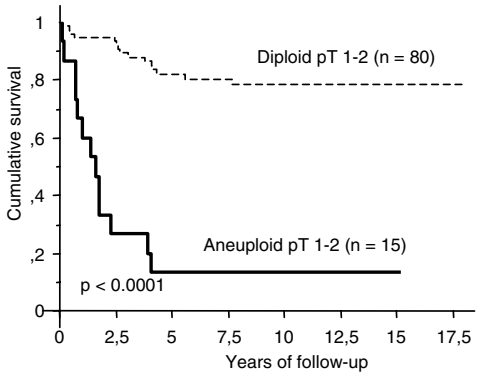

Fig. 7b

Figure 7a-b. Gastric cancer specific survival of patients with diploid vs. aneuploid tumours including all stage groups (Fig. 7a) and of patients with pT1-2 tumours (Fig. 7b). The difference in survival between the groups is significant ($p < 0.0001$).

8.2.9 SPF (V)

The median SPF of all tumours was 7.6 %. SPF could not be assessed in 40 tumours. In diploid tumours, the median SPF was 6.7 %, compared to 27.9 % in aneuploid. A statistically significant correlation was found between SPF and p53 ($r_s = 0.38$, $p < 0.0001$) and tumour size ($r_s = 0.26$, $p < 0.0001$). A high SPF (≥ 7.6 %) was associated with advanced stage ($p = 0.0003$), moderate to high TATI expression ($p = 0.0002$), intestinal type of tumours ($p < 0.0001$), non-curative surgery ($p < 0.0001$), aneuploid tumours ($p < 0.0001$), and loss of epithelial syndecan-1 ($p < 0.0001$).

Survival time was significantly shorter of patients with a high SPF than with a low SPF: the 5-year survivals were 19 % and 46 %, respectively ($p < 0.0001$; Table 5; Figure 8a). The difference was especially marked in the subgroup of patients with non-metastatic disease (pM0) with 5-year survival percentages of 30 % and 56 %, respectively ($p < 0.0001$; Figure 8b).

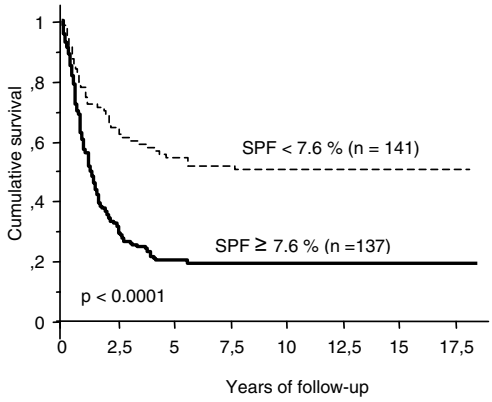

Fig. 8a

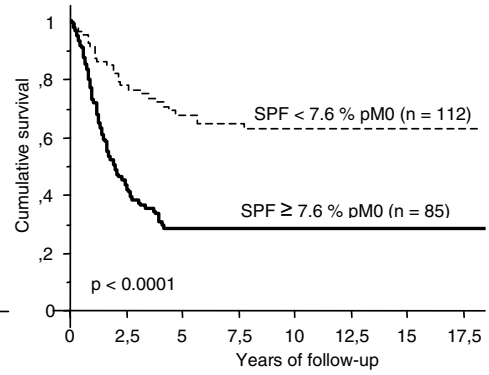

Fig. 8b

Figure 8a-b. Gastric cancer specific survival of patients with high SPF ≥ 7.6% vs. low SPF < 7.6% tumours including all stage groups (Fig. 8a) and of patients with unmetastasized pM0 tumours (Fig. 8b). The difference in survival between the groups is significant ($p < 0.0001$).

8.3 Multivariate survival analysis (V)

The combined multivariate survival analysis included all prognostic factors available. Here, penetration depth (pT) emerged as the most significant independent prognostic factor, followed by the presence of regional lymph node metastases (pN), estimated cure by surgery, p53 expression, DNA ploidy, p21 expression, and age (Table 6). The following variables did not predict independently outcome at the 5 % significance level: epithelial or stromal syndecan-1, tenascin-C, TATI, distant metastases, tumour size, SPF, gender, tumour location, and Borrmann's class. Age and tumour size were analysed as continuous variables. Tumour location was nominally coded. All the other variables were coded into categories as in Table 4 and Table 5. Entering SPF as a continuous variable did not affect the results.

Table 6. Stepwise multivariate survival analysis by Cox's proportional hazards model of prognostic covariates of survival in 285 patients with gastric cancer.

Covariate	p-value	RH	CI 95 %	β coefficient
pT status	<0.0001			
T1		1.00		
T2	0.080	2.280	0.905 - 5.746	0.824
T3	0.0015	4.113	1.719 - 9.837	1.414
T4	<0.0001	7.038	2.867 - 17.276	1.951
pN status	<0.0001			
N0		1.00		
N1	0.0004	2.232	1.426 - 3.493	0.803
N2	<0.0001	4.259	2.491 - 7.284	1.449
Resectability	0.0006	2.105	1.374 - 3.225	0.745
p53	0.004	1.582	1.158 - 2.159	0.458
DNA ploidy	0.011	1.499	1.098 - 2.047	0.405
p21	0.018	1.671	1.092 - 2.556	0.513
Age	0.031	1.014	1.001 - 1.027	0.014

RH = relative hazard. CI 95 % = confidence interval at 95 % level.
pT, pN, resectability, p53, DNA ploidy, and p21 were categorically coded (Table 4 and 5).
Age was analysed as a continuous variable.

9. DISCUSSION

9.1 Epithelial and stromal syndecan-1 expression

In paper I, loss of epithelial syndecan-1 expression and gain of stromal syndecan-1 expression were associated with an unfavourable disease specific survival (Table 5). Similar results have previously been reported for other cancers. Reduced epithelial syndecan-1 expression is associated with a decreased disease specific life expectancy in squamous cell carcinoma of the head and neck, in laryngeal cancer and in mesotheliomas (Anttonen et al., 1999; Pulkkinen et al., 1997; Kumar-Singh et al., 1998).

A low number (≤ 60 %) of syndecan-1 positive cancer cells in the tumour was associated with intestinal type of tumour (according to Laurén), deep penetration, and location of the tumour in the upper third of the stomach. Low syndecan-1 expression was also associated with large (> 5 cm) primary tumours, positive nodal status (N0 vs. N1-2), and advanced clinical stage. These finding are similar to what has been reported for head and neck carcinoma (Anttonen et al., 1999).

Syndecan-1 is expressed in the stroma of cancer tissue (Stanley et al., 1999). Syndecan-1 is strongly expressed in the stroma of infiltrating ductal breast carcinomas, but absent from the stroma of the healthy breast tissue and of stromal-epithelial neoplasms. Changes in syndecan-1 expression, induction within the stroma and reduction or loss in the malignant cells, could be critical for promoting the metastatic phenotype of infiltrating ductal carcinoma of the breast (Stanley et al., 1999). The proportion of tumours with a positive stroma was low, only 9 % (Table 5). Stromal syndecan-1 expression was associated with the intestinal type of cancer, Borrmann's type I cancer and weak (≤ 60 %) epithelial syndecan-1 expression. Patients with a syndecan-1 positive stroma had a significantly reduced survival time compared with patients who did not stain for syndecan-1 in the stroma (Table 5).

The adhesion molecule syndecan-1 can suppress tumour cell invasion and tumour growth (Mali et al., 1994). Expression of syndecan-1 in tumour cells might therefore be associated with a better prognosis, and this is, in fact, supported by the present finding. Loss of syndecan-1 on the surface of cancer cells might be a necessary condition for migration of metastatic cells. Stromal expression of syndecan-1 could be associated with loss of epithelial expression, decreased cell adhesion and degradation of basement membranes. Thus, stromal syndecan-1 positivity may be a sign of aggressive malignant behaviour (Stanley et al., 1999).

9.2 Nuclear p27 expression

In paper II, there was no difference in survival between patients with low or high p27 immunoreactivity (Table 5).

Loss of p27 expression was frequent; 95 % of the tumours had p27 expression in < 50 % of cancer cell nuclei. The corresponding percentages in previous studies have ranged from 48 to 82 %, independently of geographical regions (Kwon et al., 1999; Ohtani et al., 1999; Feakins et al., 2000; Muller et al., 2000; Sgambato et al., 2000). A low expression of p27 is a predictor of a poor prognosis of patients with various solid cancers, including breast, colorectal and oesophageal cancers (Shamma et al., 2000; Tenjo et al., 2000; Leivonen et al., 2001). However, there are also studies on oesophageal cancer that have failed to find a prognostic value for p27, as well (Itami et al., 1999). Results in gastric cancer studies are also controversial. Ohtani et al., Kwon et al. and Sgambato et al. in their studies on 225, 115 and 96 gastric cancer patients, respectively, found that loss of p27 is an independent predictor of poor survival by multivariate analyses (Kwon et al., 1999; Ohtani et al., 1999; Sgambato et al., 2000). There is also an association between p27 expression and depth of tumour invasion (Ohtani et al., 1999), lymph node metastases (Ohtani et al., 1999), poorly differentiated histology (Ohtani et al., 1999), diffuse type of tumours (Kwon et al., 1999), and lymphatic invasion (Ohtani et al., 1999). In study II there were no significant associations between the expression levels of p27 and the common clinicopathological variables (age, gender, TNM stage of disease, grade of differentiation, Laurén's class,

Borrmann's class, depth of cancer invasion, lymph node, distant metastases). This observation is in concordance with two earlier studies on gastric cancer. Feakins et al. found no evidence of an association between p27 immunoreactivity and gastric cancer specific outcome or stage, age, gender, tumour differentiation or histological type in a series of 71 gastric cancer patients (Feakins et al., 2000). Muller et al. in their study on 413 patients who had undergone potentially curative surgery observed no independent prognostic value of p27 expression by multivariate survival analysis, nor did they register any correlation between p27 expression and the common prognostic parameters (depth of invasion, lymph node metastases, blood or lymphatic vessel invasion, type, WHO, Laurén's class) (Muller et al., 2000).

9.3 Stromal tenascin-C expression

Gastric cancer patients with strong tenascin-C immunoreactivity in the tumour stroma had a significantly longer cancer specific survival than patients with no or moderate tenascin-C expression (Table 5; Figure 5) as shown in paper III. Strong stromal tenascin-C expression was more common in tumours of the distal third of the stomach, in superficial tumours with penetration from mucosa to subserosa (pT1-2) and in tumours of clinical TNM stage I. In a previous report on 203 patients with gastric cancer there was a strong correlation between tenascin-C expression and intestinal type of cancer (Zirbes et al., 1999), but the present study did not identify such an association.

Stromal tenascin-C expression has been associated with a better overall survival of patients with cancer, whereas epithelial tenascin-C may be tumour growth promoting (Sakakura & Kusakabe, 1994; Ishihara et al., 1995). The present results confirm earlier findings in colorectal (Sugawara et al., 1991; Iskaros et al., 1997), breast (Shoji et al., 1993) and cervical (Pilch et al., 1999) cancer studies, where patients with tenascin-C expression in the tumour stroma have had a longer cancer specific survival than patients with reduced stromal expression of tenascin-C. Among the present patients, the cancer specific survival increased with increasing stromal tumour tenascin-C immunoreactivity (Figure 5). However, tenascin-C correlated strongly with TNM stage

and therefore did not emerge as a prognostic factor independent of stage in multivariate survival analysis. Three previous studies on gastric cancer patients have not either shown any prognostic value of tenascin-C (Ikeda et al., 1995; Ilunga & Iriyama 1995; Zirbes et al., 1999). In one of these studies the same antibody was used as in the present one (DB7), but the antigen retrieval method was different (Ikeda et al., 1995). This patient series included only 85 surgically treated gastric cancer patients and reported a much lower rate of tenascin-C expression. The immunohistochemical methods in the other two studies differed from that of the present one by antibody or antigen retrieval (Ilunga & Iriyama 1995; Zirbes et al., 1999).

Tenascins contain a large number of fibronectin type III repeats which vary in different tenascin-C splice variants (Chiquet-Ehrismann et al., 1994). Different splice variants may have various functions (Ghert et al., 2001). In cell culture studies, for example, purified tenascin-C can promote cellular adhesion or detachment as well as stimulate or inhibit cell division, probably due to activities of the different domains of the molecule (Erickson, 1993; Faissner et al., 1994). A recent in vitro study has shown different adhesive properties between different tenascin-C splice variants (Ghert et al., 2001). Large differences in the diagnostic value of distinct tenascin-C MAbs have also been reported (Dueck et al., 1999). These findings could explain some of the contradictory results in gastric cancer prognostic studies. Epithelial tenascin-C might support the outgrowth of carcinoma cells, whereas stromal tenascin, by covering the cancer nest, might hinder cancer invasion (Sakakura & Kusakabe, 1994; Ishihara et al., 1995). In paper III, stromal tenascin-C expression was significantly associated with penetration depth: expression was less common in tumours penetrating to the serosa and adjacent structures. These findings support the hypothesis that tenascin-C may be a tumour invasion-limiting factor. If this is the case, this finding may have important clinical implications, also suggested previously (Sugawara et al., 1991).

9.4 Tissue expression of tumour-associated trypsin inhibitor (TATI)

Expression of TATI was present in 79 % of the analysed tumours in paper IV. Patients who had a tumour with high immunohistochemical expression of TATI had a

significantly longer cancer-specific survival than those with negative, low or moderate expression (Table 5; Figure 6).

TATI, identical to pancreatic secretory trypsin inhibitor PSTI, is a strong inhibitor of trypsin (Fritz et al., 1967; Huhtala et al., 1982; Turpeinen et al., 1988). TATI and trypsin are co-expressed in neoplasms (Solakidi et al., 2003). TATI is expressed in mucus producing cells of the normal gastric mucosa and secreted into the gastric juice probably preventing proteolytic digestion of the gastric mucus (Freeman et al., 1990; Playford et al., 1991; Marchbank et al., 1998). In atrophic gastritis, stomach ulcers and areas of intestinal metaplasia the expression of TATI is decreased, which could be a sign of a decreased mucosal defence mechanism that might allow progression of the gastric process over time (Bohe et al., 1987; Playford et al., 1994). PSTI/TATI may also stimulate repair after mucosal damage (Marchbank et al., 1998). The results of the present study support a protective role of TATI against tumour invasion. A high TATI expression associated statistically significantly with low TNM stage, superficial tumours, lack of nodal and distant metastasis, and high expression of epithelial syndecan-1 in paper IV.

However, the associations are contradictory to those between serum levels of TATI and indicators of tumour aggressiveness. In gastric cancer, elevated serum levels of TATI are associated with advanced disease stage (Loizate Toricaguena et al., 1991; Piantino & Arosaio, 1991). An increase in serum levels of TATI is a prognostic marker and indicator of an unfavourable prognosis of patients with ovarian, bladder and renal cell cancer (Venesmaa et al., 1994; Venesmaa et al., 1998; Paju et al., 2001; Kelloniemi et al., 2003). The association between a poor outcome of patients with high serum levels of TATI may be explained by degradation of basement membranes in aggressive disease with loss of tissue architecture and an increased release of TATI into the circulation. This would explain the discrepancy between the current findings that high TATI expression in tumour tissue is associated with a better prognosis (Table 5) and that high levels of TATI in serum associates with a poor prognosis (Loizate Toricaguena et al., 1991; Piantino & Arosaio, 1991; Venesmaa et al., 1994; Venesmaa

et al., 1998; Paju et al., 2001; Kelloniemi et al., 2003). In a previous report on gastric cancer, tissue expression of PSTI/TATI was associated with advanced TNM stage, deeper penetrating tumours and nodal involvement in intestinal type of tumours (Higashiyama et al., 1990). The overall percentage of positive cancer cells (83 %) was similar to that in the present study (79 %). There are certain differences between the studies that could explain the discrepant results. The antibody, a polyclonal PSTI antiserum, differed from the monoclonal antibody used in the present study, which has proven to be highly sensitive for TATI (Osman et al., 1993). In the present study, a four-level scoring was used, whereas PSTI expression in the previous study was analysed only in terms of positive or negative. The earlier study presented no prognostic data. In a study on patients with ovarian cancer, TATI tissue expression was associated with adverse cancer specific survival (Paju et al., 2004). However, this was found to be the case only in a limited number of patients with stage III and IV cancer.

In paper IV, the prognosis was significantly more favourable for patients with high TATI expression in the tumour tissue when compared to the groups with negative, low or moderate TATI expression, separately or as a group (Table 5). The difference in survival was not significant between the groups with moderate, weak, or negative TATI expression and hence the TATI cut-off level was set high for purposes of statistical analysis (> 50 % of cancer cells positive). Interestingly, the prognosis of patients with TATI negative tumours was similar to the prognosis as of patients with moderately stained tumours. This suggests a protective role for TATI against tumour invasion, possibly by reducing the proteolytic activity of trypsin and thereby by inhibiting tissue destruction and mucosal degradation.

9.5 Combined analysis of previous markers and p53, p21, bcl-2, DNA ploidy, and SPF

Although some markers turned out to be prognostically independent of TNM stage or the T, N and M factors, it is important to run multivariate survival analyses and to combine information from several tumour markers (Gospodarowicz et al., 2001; Partridge et al., 2005). In paper V , p53 and p21 expression, and DNA ploidy emerged

as independent markers of patient prognosis, in addition to depth of penetration (pT), regional nodal status (pN), estimated cure by surgery, and age (Table 6).

These results are in concordance with several gastric cancer studies that identify p53 as a prognostic factor (Martin et al., 1992; Joypaul et al., 1994; Starzynska et al., 1996; Victorzon et al., 1996a; Ichiyoshi et al., 1997; Ikeguchi et al., 1998; Setälä et al., 1998). The other independent prognostic tumour marker was p21, which is activated by wild-type p53 and inhibits progression of the cell cycle (Cayrol & Ducommun, 1998). There is, however, evidence that p21 is also a major inhibitor of apoptosis, which may or may not be dependent on p53, but the exact pathophysiological mechanisms are unclear (Cayrol et al., 1998; Gartel & Tyner, 2002). Some reports claim that p21 is a predictor of an unfavourable outcome (Gomyo et al., 1997; Liu et al., 2001; Xiangming et al., 2000; Aoyagi et al., 2003), but others have failed to show any prognostic value of p21 to gastric cancer patients (Muller et al., 1999; Kaye et al., 2000).

DNA ploidy was also an independent predictor of survival here, as has been reported previously in a different series of gastric cancer patients (Victorzon et al., 1996b). SPF was not an independent prognostic factor by multivariate survival analysis, probably because of its strong association with DNA ploidy. The traditional prognostic indicators, penetration depth (pT) and regional nodal status (pN), remained the strongest predictors of survival. Because the pM-status associated strongly with the type of surgery (with or without curative intent), it did not emerge as an independent factor. The markers syndecan-1, tenascin-C, and TATI were not significant prognostic markers after adjustment for the above-mentioned factors in paper V. Nor did the markers bcl-2 and p27 show prognostic value by univariate survival analysis; they were not included in the multivariate analysis.

10. CONCLUSIONS

In gastric cancer patients:

1. loss of syndecan-1 in the epithelium in surgically treated patients is associated with a poor outcome. Expression of syndecan-1 in the stromal tissue is also associated with a poor outcome and seems to be a prognostic factor independent of tumour stage, estimated cure by surgery, and tumour size.

2. the expression of p27 protein in the tumour tissue is not associated with outcome. There was no correlation between expression of p27 and the conventional clinicopathological variables age, gender, stage of disease, depth of cancer invasion, lymph node or distant metastases, grade of differentiation, histological type according to Laurén, or Borrmann's class.

3. strong stromal expression of tenascin-C is associated with a favourable prognosis. Tenascin-C expression associates with the depth of tumour invasion and may not add significant prognostic information to that provided by TNM stage.

4. a high expression of TATI in tumour tissue is associated with a favourable prognosis, although TATI did not turn out to be an independent predictor of survival by multivariate analysis.

5. p53 and p21, but not bcl-2, have independent prognostic value: high expression is associated with a worse prognosis. Patients with aneuploid tumours and tumours with a high SPF have an unfavourable prognosis.

6. multivariate survival analysis showed that p53 expression, p21 expression, and DNA ploidy are independent prognostic variables, in addition to the conventional variables pT- and pN-status, estimated cure by surgery, and age. Epithelial and stromal syndecan-1, stromal tenascin-C and TATI immunoreactivity did not add significant prognostic information in the combined multivariate survival analysis.

11. SUMMARY

The estimated prognosis of patients with gastric cancer is important, not only for the patient, but also for the planning of treatment. The most established prognostic factor is the UICC TNM-stage classification. However, there is a considerable variation in survival of patients within the separate stage groups and there is a need for other prognostic markers to improve the accuracy of prognostication.

This study used immunohistochemistry to assess the expression of tumour markers involved in different phases of tumourigenesis: markers of cell-cell (syndecan-1) or cell-extracellular matrix (tenascin-C) adhesion, cell-cycle regulation (p27, p21, p53), proteinase inhibition (TATI), and apoptosis (p53, p21, bcl-2). The DNA ploidy status and S-phase fraction (SPF) of the tumours as markers of proliferation were assessed by flow cytometry. The final aim was to identify novel prognostic markers in gastric cancer and to test them with the classical prognostic factors in a multivariate model to improve the accuracy of prognosis assessment for patients with gastric cancer.

Tumour markers that associated with an unfavourable prognosis in univariate survival analysis were loss (≤ 60 % of the cancer cells surface stained) of syndecan-1 expression, stromal syndecan-1 immunoreactivity, low stromal tenascin-C intensity, low (≤ 50 % of cancer cells stained) TATI expression, high (> 20 % of cancer cell nuclei stained) p53 expression, high (> 20 % of cancer cell nuclei stained) p21 expression, aneuploidy, and a high (≥ 7.6 %) SPF. Other characteristics associated with unfavourable survival in univariate analysis were, in order of significance, high TNM stage, non-curative surgery, regional lymph node metastases, deep penetrating tumours, presence of distant metastases, large tumour size, tumour location in the upper third of the stomach, linitis plastica (Borrmann's type IV), and advanced age. There was no statistically significant difference in survival between patients by gender or by Laurén's classification (intestinal or diffuse type) of tumours. The cell-cycle inhibitor p27 and the apoptosis inhibitor bcl-2 did not turn out to have prognostic value.

Multivariate survival analysis adjusted for all prognostic variables available showed that the most significant variables were penetration depth (pT) and the presence of

regional lymph node metastases (pN), followed by curative intent of surgery, p53 expression, DNA ploidy, p21 expression, and age. As an example, the risk of death due to gastric cancer of a patient with a cancer of pT4 (RH 7.038), pN2 (RH 4.259), high p53 expression (RH 1.582), aneuploidy (RH 1.499), high p21 expression (RH 1.671), non-curative surgery (RH 2.105), and one year more age (RH 1.014), is 19.2 times higher than for a one year younger patient with a pT1, pN0 tumour with weak p53 and p21 expression, diploidy, and curative surgery. Although syndecan-1, tenascin-C, and TATI were promising prognostic markers by univariate survival analysis, they provided no significant prognostic information in the final combined multivariate survival analysis.

Continuous advances in molecular biology call for consensus concerning the processes and standardisation of immunohistochemical, flow cytometric and other prognostic studies to maximise accuracy and reproducibility to the benefit of gastric cancer patients. These topics must be periodically revisited and revised. Currently, the methods are largely suited for research only, since the study techniques and results of different clinical centres vary widely. Although p53, p21 and DNA ploidy emerged as independent prognostic factors in this study, there is a need for more and larger studies before any of these variables can be considered evidence based and taken into clinical use.

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